

NON-SPECIFIC RECOGNITION BY MACROPHAGES
AND MECHANISMS OF MACROPHAGE ACTIVATION

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This work is dedicated to the memory of my father, Ögmundur
Jónsson, who died shortly before this thesis was completed.

Nature has provided, in the white corpuscles as you call them -
in the phagocytes as we call them - a natural means of devouring
and destroying all disease germs. There is at bottom only
one genuinely scientific treatment for all diseases, and that
is to stimulate the phagocytes. Stimulate the phagocytes.

Bernard Shaw "The Doctor's Dilemma", 1906.

ABSTRACT

This study deals with two related aspects of macrophage function, surface recognition and signal transmission across the plasma membrane.

The introduction reviews the properties and functions of macrophages with particular reference to specific and non-specific recognition and activation as expressed by enhanced effector function. This review is set against the background of the structure and function of plasma membranes, biological recognition and mechanisms of cellular activation.

The ability of macrophages to recognize a variety of foreign particles without the mediation of specific recognition molecules was investigated. In a binding assay performed at 4°C using non-opsonized bacteria, it was found that several types of Gram-positive and Gram-negative bacteria bound to normal mouse peritoneal macrophages. The binding could be inhibited by pre-incubating the macrophages at 4°C with various monosaccharides at a concentration of 10 mM. There was a very close correlation between the ability of a sugar to inhibit binding of a particular type of bacterium and the presence of that sugar in the bacterial cell wall. It was, therefore, postulated that the binding of non-opsonized bacteria by macrophages was based on the recognition of cell wall carbohydrates.

The nature of the binding reaction was further studied

using Corynebacterium parvum. It was found that binding at 4°C depended on the presence of both Ca⁺⁺- and Mg⁺⁺-ions whilst binding at 20°C occurred to some degree when only Mg⁺⁺-ions were present. The binding was not mediated by cell-bound antibody as shown by experiments using mild trypsin-treatment and specific antibody. Pre-treatment of the macrophages with trypsin, pronase, β-galactosidase and phospholipases A, C and D caused a marked reduction in binding whilst treatment with neuraminidase resulted in some increase in binding. Exposure of the macrophages to periodate also led to a decrease in binding of C. parvum, an effect largely reversed by subsequent treatment with borohydride. Recovery from the effects of enzyme treatment was rapid, but was inhibited by EDTA in the case of trypsin and β-galactosidase. These results suggested that plasma membrane glycoproteins played an important part in the binding reaction which might involve a bridging action of divalent cations. The effect of neuraminidase was most easily explained by a reduction in cell surface negative charge.

The enhancement of phosphatidylinositol turnover was investigated as a possible mechanism of signal transmission initiating the intracellular effects of an activating agent following contact with the macrophage surface. The rate of phosphatidylinositol turnover was assayed by measuring the uptake of tritiated myo-inositol into macrophage phosphatidylinositol during one hour. It was shown that two macrophage

activating agents, endotoxin and C. parvum, caused an increase in phosphatidylinositol turnover after 4 hours of incubation whilst exposure to three inert particles, Staphylococcus albus, latex and colloidal carbon, had no such effect. All the particles tested were phagocytosed and it was concluded that enhanced turnover of phosphatidylinositol was an early event following exposure to activating agents that was not linked to the process of phagocytosis.

Prolonged exposure to endotoxin resulted in enhanced bacteriostatic activity of the macrophages against Listeria monocytogenes. C. parvum and endotoxin also stimulated lysosomal enzyme activity of the macrophages. There was, thus, a correlation between the ability to stimulate phosphatidylinositol turnover and to induce the macrophage effector functions characteristic of the activated state.

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This study has been designed and carried out by the author. During the period of this research project papers on several aspects of the work have appeared in scientific journals as noted below. These are included in this thesis as an appendix.

List of publications from this work:

- 1) ÖGMUNDSDÓTTIR, H.M. & WEIR, D.M. (1976). The characteristics of binding of Corynebacterium parvum to glass-adherent mouse peritoneal exudate cells. Clin. exp. Immunol., 26, 334-339.
- 2) WEIR, D.M. & ÖGMUNDSDÓTTIR, H.M. (1977). Non-specific recognition mechanisms by mononuclear phagocytes. Clin. exp. Immunol., 30, 323-329.
- 3) ÖGMUNDSDÓTTIR, H.M., WEIR, D.M. & MARMION, B.P. (1978). Binding of micro-organisms to the macrophage plasma membrane; effects of enzymes and periodate. Brit. J. exp. Path., 59, 1-7.
- 4) FREIMER, N.B., ÖGMUNDSDÓTTIR, H.M., BLACKWELL, C.C., SUTHERLAND, I.W., GRAHAME, L. & WEIR, D.M. (1978). The role of cell wall carbohydrates in binding of microorganisms to mouse peritoneal exudate macrophages. Acta path. microbiol. scand. B, 86, 53-57.

- 5) ÖGMUNDSDÓTTIR, H.M. & WEIR, D.M. (1979). Stimulation of phosphatidylinositol turnover in the macrophage plasma membrane: a possible mechanism for signal transmission. Immunology, 37, 689-696.

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INTRODUCTION

1. HISTORICAL BACKGROUND

The process of phagocytosis and the role of phagocytes in the defence against infective diseases was first described by Metchnikoff almost a century ago. He distinguished between microphages and macrophages and described the former as very actively phagocytic cells that appeared in acute inflammatory lesions and were capable of ingesting a variety of microorganisms. The macrophages on the other hand were found in older lesions and were particularly associated with certain types of organisms, including Mycobacteria, against which microphages were less effective. Macrophages also differed from microphages in their ability to phagocytose large particles, such as red blood cells and even microphages that contained ingested bacteria. Metchnikoff's long standing dispute with the proponents of the theory of humoral immunity, such as Buchner and von Behring, is well known; he accepted that certain serum factors did play a role in acquired immunity but thought their effect was mainly to stimulate the phagocytes. He also noted that non-specific stimuli, such as peptonized broth and non-pathogenic bacteria, could render macrophages more capable of dealing with pathogenic microorganisms. Metchnikoff came to the conclusion that in acquired immunity the phagocytes had become adapted to produce large quantities of bactericidal substances which might then also be found in the body fluids (see Metchnikoff, 1905).

Denys (1898) performed some experiments in vitro which demonstrated that specific serum factors from immune animals were required for the effective phagocytosis of Streptococcus pyogenes regardless of whether the phagocytes came from a normal or an immune animal. Sir Almroth Wright showed that normal serum contained a heat labile factor that could also prepare microorganisms for phagocytosis and referred to the substance(s) responsible for the effect as "opsonin(s)" (Wright & Douglas, 1903). A few years later the work of Neufeld and others (e.g. Neufeld & Bichel, 1908) led to the conclusion that Wright's opsonin was identical with complement. Neufeld's work also demonstrated the existence of both a purely humoral immune reaction, mediated by antibodies (lysins) and complement, and a combined humoral and cellular immune reaction where antibodies (tropins) prepared particles for phagocytosis.

Aschoff (1924) defined the reticuloendothelial system on the basis of the ability to take up dyes in vivo. The function of the reticuloendothelial system was thought to be the clearance from the bloodstream of bacteria, other foreign material and effete tissue elements, in particular red blood cells (Wright, 1927; Topley, 1933; Gottlieb, 1934a, b, c; Zinsser et al., 1939). Certain microorganisms were shown to owe their pathogenicity to an ability to escape phagocytosis; the uptake by phagocytes being greatly facilitated by specific antisera (Wright, 1927; Dubos, 1945). At that time phagocytosis was regarded as a

physicochemical phenomenon (Mudd et al., 1934). The cells of the reticuloendothelial system were also believed to be the producers of antibody (Topley, 1933).

The immune response in certain chronic infections, notably tuberculosis, could not be made to fit into this picture of clearance of pathogenic bacteria by antibody-mediated phagocytosis. Lurie (1942) showed that during a tuberculous infection the macrophages acquired an increased capacity to inhibit the growth of intracellular Mycobacteria and that this was expressed even in the absence of immune serum. The mechanism of immunity to this type of infection was not explained fully until Mackaness showed that the immune response to Listeria monocytogenes and other intracellular parasites was mediated by immune lymphocytes which in turn activated the macrophages to become highly microbicidal in a non-specific manner (Mackaness, 1964 & 1969). Subsequently it was demonstrated that lymphocytes exposed to antigen released factors (lymphokines), different from antibodies, that inhibited the migration of macrophages (Bloom & Bennet, 1966) and stimulated their phagocytic and metabolic activities (Nathan et al., 1971).

In the last 15-20 years there has been a renewed interest in the macrophage as a component of the immune system. The role of macrophages in the induction of the specific immune response was suggested by the studies of Fishman & Adler (1963) and Nossal & Ada (e.g. Nossal et al., 1964). Many investigators have studied the uptake of antigen by macrophages and subsequent

presentation to T- and B-lymphocytes in vivo and in vitro (e.g. Cohn, 1962; Argyris, 1967; Cline & Swett, 1968; Rosenthal et al., 1975). It has become clear that there is an absolute requirement for the presence of macrophages for the response of T-lymphocytes to antigens (Mosier, 1967; Waldron et al., 1973; Rosenstreich & Oppenheim, 1976). The same may hold true for purely B-lymphocyte mediated responses (Chused et al., 1976) although these have appeared to be less sensitive to depletion of macrophages (Pierce & Kapp, 1976). Apart from the presentation of antigen to lymphocytes, macrophages have been shown to secrete factors which promote the stimulation of T- and B-lymphocytes (Gery & Waksman, 1972; Wood & Gaul, 1974; Rosenstreich & Oppenheim, 1976). It was soon noted that, in excessive numbers, macrophages depress the activity of lymphocytes in vitro (Harris, 1965; Hersh & Harris, 1968; Hoffmann, 1970) and there are indications of the production of inhibitory factor(s) suggesting a regulatory role for macrophages (Nelson, 1973 & 1976).

Specific receptors on the macrophage plasma membrane for immunoglobulin and complement have been demonstrated by several workers (e.g. Boyden, 1964; Berken & Benacerraf, 1966; Lay & Nussenzweig, 1969a) and this has recently led to a new concept of particle attachment and phagocytosis as receptor-mediated phenomena (Mantovani et al., 1972; Griffin et al., 1975).

The first suggestion that macrophages might be involved

in the cytotoxic reaction against allografts was made by Gorer (1956). Granger and Weiser (1964) showed that macrophages could have a specific cytotoxic effect on allogeneic cells. It was several years later, however, that Alexander and Evans (1971) and Hibbs et al. (1972) demonstrated that macrophages could also be "activated" to become non-specifically cytotoxic against a variety of tumour cells and this raised the possibility that they could play an active role in the host defence against malignancies.

In the last few years the macrophages have also received attention as secretory cells. Various stimuli can induce the release of lysosomal enzymes (Davies & Allison, 1976) and certain conditions stimulate the synthesis and secretion of neutral proteinases (Unkeless et al., 1974; Wahl et al., 1974; Werb & Gordon, 1975b). More recently stimulated macrophages have been shown to release prostaglandins (Bray et al., 1974; Humes et al., 1977). These secretory products were postulated to be of potential importance in chronic inflammatory lesions (Bray et al., 1974; Davies & Allison, 1976).

It can be seen from this brief historical sketch that views on macrophage function have changed dramatically in the last 15 years or so. Macrophages are no longer regarded merely as scavenger cells; a function which is of course highly important as was recognised by the early workers. These cells are now known to be of fundamental importance in the

afferent as well as the efferent limb of the specific immune response. The implications of their different functional states induced by immunological and non-immunological pathways and reflected in the secretion of biologically active substances and effects on other cells are currently receiving a great deal of attention not least in relation to the problem of neoplasia.

2. DEFINITION

2.1 The mononuclear phagocyte system.

Macrophages belong to the mononuclear phagocyte system as defined by van Furth et al. in 1972. This classification replaced Aschoff's definition of the reticuloendothelial system which had been in use for almost 50 years (Aschoff, 1924). The cells of the mononuclear phagocyte system are defined by the following functional criteria: avid phagocytosis and pinocytosis, ability to adhere firmly to a glass surface, and phagocytosis mediated by receptors for immunoglobulin. Morphological criteria are far less reliable, they include: cell size (10-25 μ), shape of nucleus (reniform - oval), a nuclear cytoplasmic ratio of less than one for mature macrophages, and a ruffled membrane with microvilli. Cytochemical techniques have also been employed for the identification of mononuclear phagocytes. Monocytes and macrophages give a strong diffuse positive reaction for non-specific esterase (Braunsteiner & Schmalzl, 1970). T-lymphocytes also contain esterase but the staining pattern is different (Mueller et al., 1975). Peroxidase is a marker for immature mononuclear phagocytes, it is found in promonocytes and to some degree in monocytes but not in mature macrophages (Hirsch & Fedorko, 1970). The following table, taken from van Furth et al. (1972), summarizes the mononuclear phagocyte system:

CELLS	LOCALIZATION
Precursor cells	bone marrow
↓	
Promonocytes	bone marrow
↓	
Monocytes	bone marrow, blood
↓	
Macrophages	<div style="display: inline-block; vertical-align: middle;"> { <div style="display: inline-block; vertical-align: middle; text-align: left; padding-left: 5px;"> connective tissue (histiocytes) liver (Kupffer cells) lung (alveolar macrophages) spleen (free and fixed macrophages) lymph node (free and fixed macrophages) bone marrow (macrophages) serous cavity (pleural and peritoneal macrophages) bone tissue (osteoclasts?) nervous system (microglial cells?) </div> </div>

The definition of the mononuclear phagocyte system according to the functional criteria stated above excludes certain cells that Aschoff included in his reticuloendothelial system. These cells are endothelial cells, fibrocytes and reticular cells; they are only weakly phagocytic, although they take up vital dyes, and are not derived from monocytes (van Furth et al., 1972).

The table of the mononuclear phagocyte system, however, includes two cell types, albeit with a questionmark, that were not members of the reticuloendothelial system. These are osteoclasts and

microglial cells and there is evidence that both cells originate from circulating monocytes (Andersen & Matthiessen, 1966; van Furth et al., 1972).

2.2 Related cells.

In recent years several cell types have been described that share certain functions of macrophages but do not fulfil all the functional criteria of the mononuclear phagocyte system. Generally these cells have been shown either to take up antigen, interact with lymphocytes, or to have cytotoxic activity. The antigen binding dendritic cell found in lymphoid follicles and described originally by Nossal et al. (1968) is not phagocytic. Steinman and Cohn (1973 & 1975) have isolated an adherent, dendritic cell from the mouse spleen, Peyer's patches and lymph nodes that did not bind antigen or phagocytose particles but formed clusters with plasmablasts in culture. The Langerhans cells found mainly in the skin but also in the thymus and lymph nodes (Stingl et al., 1977) have recently been found to bind antigen and metals (Shelley & Juhlin, 1976) but they do not phagocytose particles. They do, however, possess receptors for complement and the Fc portion of immunoglobulin (Stingl et al., 1977). The so-called reticular cell, described by Stuart & Davidson (1971) in lymph nodes, spleen, thymus and irritated peritoneum has similar characteristics, it is dendritic and argyrophilic, glass

adherent, poorly phagocytic but it appears to take up antigen from macrophages and interact with lymphocytes (Stuart, 1977). Nathan et al. (1976) have identified cytotoxic cells in the peritoneal exudate of mice and found that these cells constitute an increased proportion of the peritoneal cell population following an injection of BCG. These cells are adherent but not phagocytic and they have now been shown to contain IgM thus suggesting that they may represent a subpopulation of B-lymphocytes (Nathan et al., 1977). Adherent but non-phagocytic effector cells in antibody-dependent cytotoxicity have been identified in the spleen (Greenberg et al., 1973) and peritoneal exudate (Allison, 1972). Recently antibody-dependent cytotoxic cells found in bone marrow, peripheral blood and inside tumours have been shown to become adherent and capable of phagocytosis after overnight culture (Haskill, 1977). A "natural" killer cell was described by Kiessling et al. (1975) and Herbermann et al. (1975) in mouse lymphoid tissue and has now also been found in the rat spleen (Oehler et al., 1978) and human peripheral blood and lymphoid organs (Eremic et al., 1978). The natural killer cell, which is directly cytotoxic against leukemia cells, has its origin in the bone marrow, it is non-adherent and non-phagocytic and has weak Fc receptor activity (Oehler et al., 1978). Eremic et al. (1978) have recently suggested that the natural killer cell may be closely related to the lymphocytic killer cell,

"K-cell", which is cytotoxic in the presence of antibody as described by Perlmann et al. (1972). Bennett et al. (1976) have reported an apparently similar cell type in the bone marrow which is active in the rejection of haemopoietic allografts and in addition seems to play a role in resistance against Listeria monocytogenes during the first few days of infection (Bennett & Baker, 1977).

Many of these cells have not been clearly characterized. They do not, by definition, belong to the mononuclear phagocyte system, but it is not clear if and how they are related to the cells of the mononuclear phagocyte system. It may be speculated that they originate from identical precursor cells, as seems to be the case for granulocytes and monocytes (Metcalf, 1971a); or that they are immature cells which have acquired some functions of mature cells but not others, as may be suggested by the results of Haskill (1977); or that they perhaps represent highly differentiated cells that are specialized to perform a specific function but have lost some of the functional abilities of younger cells. Of the different cells mentioned above the natural killer cell and the cytotoxic cell of Nathan et al. do not seem to be closely related to the mononuclear phagocytes. The existence of all these different cells has to be kept in mind when ascribing certain functions to macrophages.

2.3 Heterogeneity of macrophages.

Cells that are described as macrophages do not form a homogeneous population (Walker, 1976a). It is not known whether the sub-groups represent genuine differentiated sub-populations or cells at various stages of maturation, stimulation or activation (Rhodes, 1975). Peritoneal exudate cells can be fractionated according to bouyant density into sub-groups that differ in their ability to induce a specific immune response; their phagocytic and cytotoxic capacity; and in the expression of Fc-receptors (Rice & Fishman, 1974; Walker, 1974 & 1976b; Lee & Berry, 1977). There is also earlier work to indicate the existence of macrophages with low phagocytic and bactericidal capacity (Cooper & Houston, 1964; McIntyre et al., 1967). Direct microscopical observation has demonstrated variations in uptake of antigen correlated with cell size (Loor & Roelants, 1974). It is well known that macrophages in inflammatory exudate differ from resident peritoneal macrophages in their metabolism and enzyme activity (Karnovsky et al., 1975a) and their ability to respond to stimulation in vitro by developing cytotoxic or secretory activity (Christie & Bomford, 1975; Davies et al., 1977). Macrophages also show differences depending on their tissue location, indicating an influence of the microenvironment. Alveolar macrophages are less phagocytic and bactericidal than peritoneal macrophages but they contain higher concentrations

of lysosomal enzymes (Pavillard, 1963). They also show less chemotactic responsiveness (Ward, 1968) and Fc-receptor activity (Rhodes, 1975a) as compared with peritoneal macrophages. Both alveolar macrophages and Kupffer cells ingest and degrade antigen very efficiently but retain little in an immunogenic form as shown by in vivo transfer experiments (Cohn, 1964; Inchley, 1969). Recently, however, alveolar and splenic macrophages have been shown to be more potent accessory cells than peritoneal macrophages in in vitro stimulation of lymphocytes (Landahl, 1976; Gorenberg & Daniele, 1978). Metabolic differences between alveolar and peritoneal macrophages have been noted by Oren et al. (1963) and the former have been reported to lack a receptor for migration inhibitory factor (MIF) (Leu et al., 1972); although others have found such a receptor to be present (Moore & Myrvik, 1977).

It is noteworthy that some of the sub-groups described are poorly phagocytic (Walker, 1976a; Rice & Fishman, 1974) and could thus be regarded to fall outwith the strict definition of the mononuclear phagocytes and are reminiscent of cells such as described by Steinman & Cohn (1973 & 1975) as mentioned above. High immunogenic activity seems to be associated with low phagocytic and cytotoxic capacity (Rice & Fishman, 1974; Walker, 1976a & 1976b; Lee & Berry, 1977). These variations have to be remembered when discussing macrophage function in general.

3. PHYLOGENY

As the present study concerns itself with a non-specific and probably primitive recognition mechanism of mononuclear phagocytes it is relevant to review briefly the phylogeny of phagocytic cells.

3.1 Phagocyte function in invertebrates.

The origin of mononuclear phagocytes can be traced back to the amoeba where the entire organism is one phagocytic cell. Metchnikoff made his pioneering observations on a primitive species, Daphnia, a freshwater crustacian. He and his colleagues subsequently studied phagocytosis in many animal species, including amoebae, insect larvae, molluscs, fish, frogs, birds and mammals (Metchnikoff, 1905). Phagocytic cells have been demonstrated in species belonging to all the invertebrate phyla. In many invertebrates (e.g. sponges, coelenterates, molluscs and echinoderms) they fulfil a digestive function (Jenkin, 1976). Phagocytes in many invertebrates also take up indigestible foreign material, such as carbon, which is then carried to the exterior of the animal. Large foreign objects are encapsulated by phagocytes as Metchnikoff (1905) demonstrated when he introduced rose thorns into starfish larvae. The repair of tissue injury always involves phagocytic cells, which clear away the debris, and they play a role in the metamorphosis of many animals (see Jenkin, 1976). Phagocytes

occur both as free moving cells, referred to as amoebocytes, haemocytes or coelomocytes, and as fixed cells. The latter are often strategically situated so as to prevent the entry of foreign material into the animal or to clear the body fluids of such material, e.g. in the gills of the Octopus (Stuart, 1970) and gills and hepatopancreas of the freshwater crayfish (Reade, 1968a).

Immunity in primitive terms is concerned with two things: on one hand the elimination of multiplying microorganisms and on the other hand the maintenance of the integrity of a multicellular organism by the exclusion of "non-self" elements. In nature such "non-self" elements may be represented mainly by cells infected with viruses and multicellular parasites rather than tissue grafts although the latter do occur naturally on coral reefs (Hildemann et al., 1977). Phagocytic cells are involved in the former type of immunity in a direct and obvious way, their role in the latter may be less direct.

The phagocytes, even amoebae, frequently show a degree of selectivity in that they ingest certain types of bacteria but not others and bacteria that are not phagocytosed often cause fatal infections (Metchnikoff, 1905). Cameron (1934) studied the response to infection in the larva of the bee moth and found that some types of bacteria were phagocytosed and destroyed, others were phagocytosed but survived inside the phagocytes

and killed the host, and a few were hardly phagocytosed but seemed to be destroyed by body fluids. Phagocytosis in invertebrates is often aided by opsonizing substances present in the body fluids (Jenkin, 1976) although there are exceptions such as the cockroach, the haemocytes of which phagocytose erythrocytes and bacteria in the absence of demonstrable opsonins (Scott, 1971; Anderson et al., 1973). Little is known about the specificity of the opsonizing factors but a certain degree of specificity seems to be implied by absorption experiments using the haemolymph of the snail (Prowse & Tait, 1969) and the crayfish (McKay & Jenkin, 1970). In both cases it was found that the opsonizing factor for one kind of particle could be completely absorbed out without affecting the opsonizing activity of the haemolymph for a different particle. Some invertebrate opsonins have been shown to recognize sugars (Uhlenbruck, 1974).

Invertebrates are generally not thought to be capable of showing an adaptive, specific immune response. Non-specific stimulation of their phagocytic system does, however, seem to occur. Metalnikov showed that the cellular reaction in larvae of the bee moth to injected bacteria is accelerated following pretreatment with dead or avirulent bacteria of the same kind and the larvae survived subsequent injection with otherwise lethal doses of bacteria (Metalnikov & Gaschen, 1922; Metalnikov, 1924). Cameron (1932) also observed proliferation of phagocytic

cells in the earthworm following injection of particles. More recently McKay & Jenkin (1969, 1970) have shown that bacterial endotoxin can enhance the phagocytic ability of haemocytes from the crayfish and the clearance of bacteria from the body fluids of this animal. In the oyster a second dose of bacteriophage T2 is cleared more rapidly than the first dose. This accelerated clearance also occurs, albeit to a lesser degree, if an unrelated phage was injected the first time; thus implying non-specific as well as specific stimulation (Acton & Evans, 1968). These observations are reminiscent of non-specific stimulation of mononuclear phagocytes in mammals (see later).

Bactericidal substances also play a role in the defence of many invertebrate species against infections. They appear to be non-specific but their concentration in the haemolymph of sipunculid worms and lobsters is increased following exposure to bacteria (Evans et al., 1968, 1969). It is not known which cells produce the agglutinins, opsonins and bactericidins found in the body fluids of invertebrates (Jenkin, 1976). It may be added at this point that the late-acting components of complement have been found in the horseshoe crab and sipunculid worm (Gigli & Austen, 1971). Some of these factors are produced by macrophages in mammals (Colten, 1976). Phagocytes would thus seem likely candidates as producers of some of the antibacterial factors of invertebrates.

Rejection of xenografts is a very universal phenomenon and many primitive species also reject allografts. Hildemann et al. (1977) have recently demonstrated specific allograft rejection in a coral, giving rise to a short lived memory. The reaction did not seem to involve phagocytic cells. Allografts in echinoderms are infiltrated by host cells, some of which are phagocytes (Hildemann & Dix, 1972; Karp & Hildemann, 1976). The amoebocytes of echinoderms phagocytose injected xenogeneic amoebocytes (Reinisch & Bang, 1971) which seems to indicate direct recognition of foreignness by the amoebocytes although the involvement of other cells or their products can not be excluded in in vivo experiments. Earthworms reject xenografts and show an accelerated reaction against a second set graft (Cooper, 1976). The xenografts are encapsulated by coelomocytes and the response can be transferred to untreated worms by coelomocytes from grafted animals (Baily et al., 1970). It is not known which type of coelomocytes, phagocytic or non-phagocytic, is primarily responsible for this reaction. A recent electron microscopical study seems to show that phagocytes invade a foreign graft in earthworms and start destroying it before other cell types appear (Linthicum et al., 1977). There is, however, also some evidence for the existence of substances similar to lymphokines in earthworms (Cooper, 1976) as well as echinoderms (Hildemann & Dix, 1972).

3.2 Mononuclear phagocytes in primitive vertebrates.

The immune system of the advanced vertebrates is characterized by a high degree of specificity and longlived memory and involves the balanced interaction of several cell types including macrophages. All vertebrates are capable of producing specific antibodies of immunoglobulin structure although the most primitive species (cyclostomes) do not show a strong response (Litman, 1976). The antibody response of ectothermic vertebrates is temperature dependent and not very efficient at low temperatures (Avtalion *et al.*, 1976). C-reactive protein, a β -globulin that precipitates with pneumococcal C-carbohydrate and various extracts from fungi and worms, is found constantly in fish even though it only appears in inflammatory processes in mammals (Baldo & Fletcher, 1973). This protein may be an important non-specific opsonin as it has recently been shown to bind to mouse macrophages by way of the receptor for the Fc portion for immunoglobulin (Mortensen & Duszkievicz, 1977). It is not known when the Fc-receptor first appeared in evolution but it is conceivable that it served first as a receptor mainly for C-reactive protein and later became the mediator of specific phagocytosis by binding of immunoglobulins.

In birds and mammals the specific immune response is thought to be initiated by an interaction between lymphocytes and antigen which is presented on the surface of macrophages (or

macrophage-like cells). This takes place in the germinal centres of the lymphoid organs. The mechanism of the immune response in the toad has been studied and it has been found that antigen is taken up by phagocytic cells in Bufo marinus and subsequently localized on the cell surface. No special relationship was observed between the phagocytes and antibody producing cells and no germinal centres were formed (Diener & Nossal, 1966; Diener & Marchalonis, 1970). Turner (1970) found that injection of carbon into the toad Xenopus laevis led to an increase in the antibody response to sheep erythrocytes and he suggested that this might be due to a migration of phagocytes to the carbon containing sites. These experiments indicate that phagocytes may be involved in the initiation of the specific immune response in primitive vertebrates although they are not very conclusive. It has to be noted that the antigen used by Diener & Nossal and Diener & Marchalonis was Salmonella flagellin. The response to this antigen is relatively independent of macrophages in the mouse, as the authors point out.

3.3 Phagocytes in the evolution of the immune system.

Several years ago Burnet (1968) suggested that all the cells comprising the immune system of vertebrates had evolved from the phagocytic amoebocyte of primitive invertebrates. As regards

B-lymphocytes this hypothesis seems to be supported by the observation that the antibody-producing cells of the tortoise are phagocytic (Ambrosius, 1976). Graft rejection in invertebrates, suggestive of a primordial "T-lymphocyte" response, appears, however, to be mediated either by non-phagocytic (Hildemann et al., 1977) or phagocytic cells (Linthicum et al., 1977). In mammals B-lymphocytes show certain similarities to monocytes/macrophages, viz. receptors for Fc and C3b and adherence to nylon wool, whilst the alloantigen marker for T-lymphocytes in the mouse (Thy or θ) is also expressed on brain cells, epidermal cells and fibroblasts (see McConnell, 1975).

4. ORIGIN AND KINETICS

Resting macrophages isolated from a normal animal for experimental use in vitro (as employed in the present study) are non-dividing, mature cells. The origins and kinetics of such cells in vivo will be summarized below as well as the changes in age and maturity of macrophage populations that can occur in response to inflammatory stimuli. Functional variations will be dealt with in a later chapter.

4.1 Ontogeny.

Macrophages are derived from haematopoietic stem cells like other cells of the immune system. Cells with characteristics of macrophages have been found in human embryonic tissues as early as the 4th week of gestation and they can be seen to enter the tissues from blood vessels (Andersen & Matthiessen, 1966). In the mouse embryo cells capable of differentiating into macrophages in vitro are present in the yolk sac from the 7th to 9th day of development. These cells are more primitive than promonocytes in that they do not possess receptors for immunoglobulin. It is likely that the yolk sac itself only provides the precursor cells and that maturation does not take place there. By the 10th to 11th day recognizable promonocytes/monocytes and macrophages appear in the yolk sac (probably derived from the foetal circulation) and in the liver (Cline & Moore, 1972).

Foetal and neonatal macrophages are capable of phagocytosis and have receptors for immunoglobulin (Reade, 1968; Cline & Moore, 1972) but they are functionally immature in other respects. Full ability of macrophages to kill bacteria and respond to viral infections develops gradually in the neonatal period (Karthigasu et al., 1965; Reade, 1968b; Hirsch et al., 1970; Blaese, 1976). Macrophages from human neonates respond only weakly to a chemotactic stimulus (Weston et al., 1977). Macrophages from newborn mice are not able to collaborate in the initiation of the antibody-producing response (Hardy et al., 1973; Landahl, 1976) and this is paralleled by the lack of antibody response to certain antigens in neonatal animals. Antibody production can be induced in neonatal mice and rats by injecting adult macrophages with the antigen (Braun & Lasky, 1967; Blaese, 1976), provided the other elements of the immune system have become sufficiently mature, which seems to occur earlier than the maturation of macrophages, or by the 3rd day in the mouse (Argyris, 1968). The phenomenon of immunological unresponsiveness of neonates is complex and apart from immaturity of the different cells of the immune system it also seems to involve suppressor T-cells (Mosier & Cohen, 1975).

Hardy et al. (1973) have suggested that T-lymphocytes might induce the neonatal maturation of macrophages and point out that

competent T-cells are not present in the mouse spleen until 3 days after birth. This is not unlikely considering the role of T-lymphocytes in the activation of macrophages in cell-mediated immunity but is unsupported by experimental evidence.

4.2 Production and turnover in adult life.

In adult life monocytes and macrophages are derived from stem cells in the bone marrow (Volkman & Gowans, 1965; van Furth & Cohn, 1968). The promonocytes in bone marrow are the direct precursors of the monocytes which are released into the blood stream. More primitive precursor cells have not been clearly identified but monocytes seem to share a common ancestor with the granulocytes. Thus monocytes/macrophages and granulocytic cells always occur together in colonies grown in vivo or in vitro from single haematopoietic stem cells, monocytes/macrophages are never found in erythrocytic colonies and they do not form pure colonies themselves (Virolainen & Defendi, 1968; Metcalf et al., 1967). Metcalf (1971a) has succeeded in cultivating macrophages from single cells with the characteristics of primitive granulocytes by an in vitro transfer from early granulocytic colonies. The relative proportions of granulocytic and monocytic cells in the in vitro colonies are dependent on the cultural conditions, in particular the presence of certain growth factors derived from other cells (Metcalf et al., 1967). Macrophages take over in most cases unless the conditions are favourable for granulocytes. Recently Dexter et al. (1977)

have demonstrated that stem cell production and granulopoiesis can be maintained in vitro for weeks on a previously prepared layer of adherent bone marrow cells, composed mainly of mononuclear phagocytes but also containing so-called "giant fat cells" around which maturing granulocytes cluster. These clusters contain no colony stimulating factor (CSF), that stimulates the formation of granulocytic/monocytic colonies in vitro from progenitor cells. When CSF is added the number of blast cells and granulocytes declines and monocytes/macrophages begin to dominate. The differentiation of stem cells into monocytes and granulocytes thus seems to be influenced by the microenvironment and humoral factors as is the case for B- and T-lymphocytes (see Golub, 1977).

The kinetics of monocytopoiesis and the turnover of mononuclear phagocytes have been studied in mouse, man and rat, using injections of tritiated thymidine, by e.g. van Furth and his co-workers (van Furth, 1970), Meuret (1974) and Volkman (1976a). One stem cell gives rise to 4 monocytes (Meuret, 1974) and each promonocyte divides into two monocytes (van Furth & Diesselhof-den Dulk, 1970). The minimum emergence time, i.e. the time lag before the first appearance of labelled cells in the blood is usually found to be approximately 6 hours; the time required for DNA-synthesis in these three species is about 12 hours (Meuret, 1974; van Furth et al., 1973; Volkman,

1976a) but the whole cell cycle of a promonocyte seems to take longer in man (29 hours, Meuret, 1974) than in the mouse or rat (16-20 hours, van Furth & Diesselhof-den Dulk, 1970; van Furth et al., 1973; Volkman, 1976a). The monocytes in bone marrow or peripheral blood are generally non-dividing cells (van Furth & Diesselhof-den Dulk, 1970) or divide only at a very slow rate (Volkman, 1976a).

Monocytes do not seem to leave the bone marrow continuously as they are formed since a fraction of labelled cells is found in the blood one hour after injecting tritiated thymidine but the majority of labelled cells appears to stay in the bone marrow for a few hours (van Furth & Diesselhof-den Dulk, 1970). After the monocytes leave the bone marrow their half-time in the circulation is about 22 hours in the mouse (van Furth & Cohn, 1968), 14 hours in the rat (Whitelaw & Batho, 1972) and 8 hours in man (Meuret, 1974). It must be mentioned, however, that figures up to or over 100 hours have been arrived at using different experimental methods and calculations (see Volkman, 1976a). Monocytes leave the circulation at random, unrelated to their age (van Furth & Diesselhof-den Dulk, 1970). On leaving the circulation the monocytes stay for about 12 hours in the margins of the blood vessels thus forming a marginal monocyte pool analogous to the marginal granulocyte pool (Whitelaw & Batho, 1972). It is generally believed that monocytes leave the blood to become tissue macrophages (van Furth et al., 1972;

Spector, 1977; van Furth et al., 1977). After i.v. injection of labelled monocytes they were found a few hours later in the spleen, lungs, liver and lymph nodes, but not in the peritoneum. Labelled monocytes did not recirculate (Whitelaw & Batho, 1972). Van Furth & Cohn (1968) recovered labelled macrophages from the peritoneum after injections of tritiated thymidine and concluded that these cells were derived from peripheral blood monocytes (which in turn had come from the bone marrow) since the uptake by peritoneal macrophages themselves in vitro was far too low to account for the labelling index observed. The low rate of uptake of tritiated thymidine by peritoneal macrophages was confirmed by van Furth et al. (1973) by pulse labelling in vivo. Volkman (1976b) however argues that the slow rate of division of tissue macrophages is sufficient for self-renewal and that recruitment from blood monocytes only occurs in inflammation. Using parabiotic rats he was not able to recover labelled cells from the peritoneal cavity of one animal after injecting its parabiotic partner with tritiated thymidine. This experiment has, however, been repeated yielding the opposite result (Parwaresch, quoted by van Furth, 1977). Time factors and varying efficiency of circulation between the parabiotic partners might account for these contradictory results. Mitotic figures are not usually observed in resting peritoneal macrophages although Daems & Brederoo (1973) report a frequency of 0.5 -

1% among resident peritoneal macrophages from guinea pigs. It seems likely that the truth lies somewhere between the two extremes and that both local division and recruitment from peripheral blood contribute to the renewal of tissue macrophages. The turnover time of tissue macrophages was estimated by van Furth (1970) to be 1-2 months, based on the assumption that they are renewed from the blood.

4.3 Maturation.

Cells of the monocyte/macrophage series mature more quickly than granulocytic cells in in vitro cultures from bone marrow and acquire receptors for the Fc-portion of immunoglobulin, C3b and C3d components of complement as well as the ability to phagocytose within 3 days. In granulocytes there is a definite order of development of these characteristics over a period of a few weeks (Rabellino et al., 1978). Promonocytes are not as actively phagocytic as monocytes and the Fc-receptor can only be demonstrated on one sixth of the promonocytes (Cline & Moore, 1972). Monocytes must acquire the Fc-receptors and both types of complement-receptors before leaving the bone marrow as all these markers are found on almost all monocytes in peripheral blood (Rabellino et al., 1978). As the monocytes mature the Golgi apparatus becomes less prominent than it was in the promonocytes, the plasma membrane becomes more ruffled and the number of surface microvilli increases. The enzyme peroxidase

is gradually lost in maturing monocytes and is not found in tissue macrophages (van Furth et al., 1970). The final maturation takes place in the tissues, with full development of the defining characteristics of macrophages, viz. strong adherence to glass, avid phagocytosis, prominent membrane ruffling and microvilli and a large number of lysosomes (van Furth et al., 1972). These tissue macrophages are in a resting state and differentiation into "activated" macrophages requires further stimulation (see e.g. Mackaness, 1970a; Davies et al., 1977; Keller, 1977).

4.4 Responses to inflammatory and antigenic stimulation.

The mononuclear phagocyte system responds to inflammatory and antigenic stimuli by an increased production of cells and migration to the site of stimulation. Monocytes accumulate at the site of acute inflammation and become transformed into macrophages. The monocyte pool is enlarged by an increase in the number of promonocytes and a reduction of their cell cycle time by about a half (van Furth et al., 1973; Volkman, 1976a). Monocytes are also released from the bone marrow in less mature form than normally found in the circulation (Volkman, 1976a). The contribution made by local division of resident macrophages is negligible in the inflammation caused by an i.p. injection of newborn-calf serum (van Furth et al., 1973).

North (1970) found that Listeria monocytogenes-infection in mice caused both an increased mitotic activity of tissue macrophages and influx of blood monocytes newly derived from precursors. The cells recruited from blood accumulate in the infective foci, become "activated" (see later) and seem to be mainly responsible for combatting the infection locally whereas resident macrophages might play a role in terminating the bacteraemia. Injection of antigen (xeno- or allogeneic red blood cells) also induces an increase in the number of macrophage-precursors in the mouse spleen as estimated by the number of colonies formed in vitro (Pluznik et al., 1972).

After the initial proliferation and recruitment the subsequent development depends on the nature of the stimulus. The effect of i.p. injected newborn-calf serum subsides after 3-4 days (van Furth et al., 1973). The response to Listeria monocytogenes declines rapidly after elimination of the bacteria from the host about the 10th day after infection (North, 1970). Irritants that can not be digested completely lead to the formation of chronic granulomas. According to Spector & Ryan (1970) the macrophage population in these chronic lesions can be maintained by three different mechanisms. Some micro-organisms, such as Bordetella pertussis cause granulomas in rats with a high turnover of cells maintained almost completely by recruitment from the bone marrow. Granulomas induced by glass coverslips show a high degree of local proliferation although

they also require augmentation from the blood. Finally there are granulomas caused by carrageenan where there is very little proliferation or recruitment but the cells in the lesion are very long-lived. When a turnover of cells occurs in a granuloma there must be a certain amount of death and removal of macrophages. Some of them may leave via the lymphatics and go to local lymph nodes but a large proportion probably dies inside the lesion (Spector, 1977). This may sometimes be caused by the ingestion of toxic material but macrophage mitosis itself often leads to chromosomal abnormalities and cell death. Old cells with abnormal nuclei often fuse with newly arrived normal cells to form giant cells, a familiar feature of chronic granulomas. The nuclei of these giant cells may undergo mitotic divisions but abnormalities are frequent and their life span is limited to a few days (Mariano & Spector, 1973). The formation of giant cells seems to be a mechanism of limiting uncontrolled division of macrophages which occurred if the arrival of new cells into the lesion was prevented (Mariano & Spector, 1973).

4.5 Regulation of production and turnover by humoral factors.

The proliferation and recruitment of mononuclear phagocytes seems to be controlled by humoral factors but the information about the factors or mechanisms involved is still fragmentary. Factors that are chemotactic for macrophages are derived from the complement system and probably also from the kinin and clotting

systems and sensitized T-lymphocytes release a monocyte chemotactic factor as well as migration inhibition factor (Snyderman & Mergenhagen, 1976).

Several other substances and microorganisms or their products are themselves chemotactic for macrophages (Snyderman & Mergenhagen, 1976; Wilkinson, 1976b). Chemotactic activity can thus be generated with or without involvement of a specific immune response.

Several years ago factors were described which induced the formation of monocyte/granulocyte colonies from precursor cells in the bone marrow (colony stimulating factor, CSF) (Metcalf et al., 1967) and some years later factors causing proliferation of resting macrophages in vitro were discovered (macrophage growth factor, MGF) (Mauel & Defendi, 1971). These factors can be obtained from a variety of sources including supernatants from various cell types, serum and urine of man and animals and inflammatory exudates. Recent evidence suggests that CSF may be identical with MGF (Stanley et al., 1976). Macrophages themselves have been shown to be a potent source of CSF/MGF and the production is increased following stimulation with endotoxin (Eaves & Bruce, 1974) or antigen (McNeill, 1970; Metcalf, 1971b). This seems to provide a positive feedback mechanism whereby macrophages that are stimulated locally or attracted by chemotaxis release a signal which increases

proliferation of mononuclear phagocytes and these would then accumulate in the inflammatory site because of chemotactic activity. Van Furth et al. (1977) have recently obtained a factor from the serum of mice in the early stages of an inflammatory response which induces the release of monocytes from bone marrow (factor inducing monocytosis, FIM). This factor appears to be released from peritoneal exudate macrophages after the induction of a peritonitis.

There is also the suggestion of a negative feedback mechanism mediated by prostaglandin E which is secreted by macrophages particularly after stimulation, including exposure to CSF, and causes a decreased responsiveness to CSF (Humes et al., 1977; Kurland et al., 1978). This negative feedback might be modulated by the increased conversion by activated macrophages of prostaglandin E2 to prostaglandin F2, which could act as a competitive inhibitor of prostaglandin E2 (Farzad et al., 1977). It may be speculated that the balance between stimulatory and inhibitory factors and feedbacks determines the chronicity of an inflammatory lesion.

The various factors mentioned above have been mainly studied in in vitro models and their function and relevance in vivo remains to be established.

5. MORPHOLOGY

5.1 Light microscopy: fixed and stained preparations.

In histology textbooks (e.g. Bloom & Fawcett, 1969) the appearance of monocytes in a smear of human blood, stained with Wright's stain, is described as follows: these cells, which constitute 3 to 8 percent of the leukocytes are larger than lymphocytes, about 9-12 μm in diameter. The eccentrically placed nucleus is oval or kidney-shaped and less intensely stained than lymphocyte nuclei. The cytoplasm is far more abundant than in lymphocytes and stains greyish blue. Tissue macrophages are described as irregular in outline, often fusiform or stellate, with an oval nucleus and heterogeneous cytoplasm, often containing granules and small vacuoles.

Hirsch & Fedorko (1970) have studied and described the appearance of the various developmental stages of mouse mononuclear phagocytes. Promonocytes are larger (15-20 μm) than more mature forms, the nucleus takes up relatively more space and the cytoplasm contains fewer granules and vesicles. Freshly isolated normal peritoneal macrophages have a diameter of about 10-14 μm and are round in outline. When macrophages are allowed to adhere to glass and then cultured in the presence of 20% heterologous serum characteristic morphological changes occur. The cells spread on the surface, increase in size and assume a stellate shape. After 4 days in culture the cells are

up to about 25 μm , irregularly stellate in outline. Binucleate forms may be seen. The abundant cytoplasm is granular and shows many clear areas, mainly at the cell periphery, probably representing lipid droplets. Normal alveolar macrophages are larger than peritoneal macrophages, about 20 μm . The cytoplasm often contains dark carbon deposits. Peritoneal macrophages that are collected 4 days after an i.p. injection of thioglycollate broth are larger than normal macrophages immediately after collection, and have an irregular shape. The cytoplasm is very heterogeneous with irregularly stained lumps and granules.

5.2 Phase contrast microscopy.

The appearance of living and fixed monocytes and macrophages under phase contrast has been described by Cohn (1968). Living monocytes exhibit a spreading, ruffled membrane in continuous motion, forming phase-lucent pinocytic vacuoles. The cytoplasm contains phase-dense granules. Living macrophages show marked activity of the cell membrane with ruffles in wave-like motion. In glutaraldehyde-fixed preparations the membrane ruffles are also seen. In the perinuclear cytoplasm there are phase-dense granules and lucent vacuoles and mitochondria, often rod-shaped, are seen both in this region but particularly in the peripheral cytoplasm and pseudopods. Lipid droplets and phagosomes are commonly seen, the number and appearance of the latter varies with the nature of ingested material. Cohn &

Benson (1965a) followed the morphological changes that occur in peritoneal macrophages after stimulation in vitro with 20% newborn-calf serum or in vivo with bacterial lipopolysaccharide. Concomitant with an increase in size and change in shape to a flattened appearance with numerous processes there was an accumulation of mitochondria, lipid droplets and phase-dense, neutral red-positive granules which contain acid phosphatase and correspond to secondary lysosomes. Secondary lysosomes are formed by the fusion of primary lysosomes, containing enzymes, with phase-lucent pinocytic vesicles, phagocytic vacuoles or occasionally vacuoles derived from the process of autophagy that contain various cytoplasmic constituents (Cohn, 1970a).

The study of Hirsch & Fedorko (1970) also included examination of different types of living mononuclear phagocytes. Surface ruffles and cytoplasmic projections become more prominent as the cells mature and monocytes and macrophages contain more phase-dense granules than promonocytes do. As the macrophages take on a flattened, dendritic shape during culture with 20% serum the perinuclear cytoplasm fills up with granules and vesicles whilst the peripheral cytoplasm in the extensions contains only mitochondria. Thioglycollate-induced cells contain huge vacuoles. The surface projections on alveolar macrophages are relatively small but these cells contain a larger number of phase-dense granules and lucent vacuoles than peritoneal macrophages.

The process of settling on a surface has been studied by Carr & Carr (1970). This process takes place in four stages. During the first stage of adhesion the cells are thick and dense with many fine projections. Subsequently the cells flatten out and show flap-like ruffles. There then follows the stage of movement with cell processes waving to and fro as well as translatory movement at a rate of 1-12 $\mu\text{m}/\text{min}$. Finally the cells reach the fully extended state, when active translatory movement has stopped and the appearance is thin and stellate. The whole process begins earlier in cells that have been stimulated in vivo but is not quicker. Fully extended cells are rarely seen until at least 5 hours after plating out normal peritoneal macrophages in Hanks' solution but are common already after 3-5 hours in preparations of stimulated cells.

5.3 Electron microscopy.

Several workers have undertaken detailed studies of the morphology of mononuclear phagocytes from mice or guinea pigs using electron microscopy (e.g. Carr, 1967; Cohn, 1968; Hirsch & Fedorko, 1970; Daems & Brederoo, 1973). Certain features are common to all mononuclear phagocytes. They all have finger-like projections and irregular contour. The nucleus contains 1-2 nucleoli. The Golgi apparatus surrounds the centrioles in the hilar region of the eccentrically placed nucleus. Granules of varying density and mitochondria are mainly scattered in an area close to the Golgi apparatus, but elongated mitochondria

are also found in the centre of pseudopods lying parallel to their axis. Many of the dense granules represent secondary lysosomes. Fat droplets, polyribosomes and rough endoplasmic reticulum are present in the outer cytoplasm, particularly on the side of the nucleus opposite the Golgi apparatus. Small vesicles are found both in relation to the Golgi apparatus and beneath the plasma membrane where they are of endocytic origin. Large vacuoles which may appear empty or filled with amorphous material are also present. Microtubules have been described in relation to the centrioles (Carr, 1967; Daems & Brederoo, 1973) as well as in the peripheral cytoplasm (Cohn, 1968). Fibrillar structures can be seen scattered throughout the cytoplasm (Carr, 1967; Cohn, 1968; Daems & Brederoo, 1973) but also in the centre of cytoplasmic projections and in an area immediately beneath the plasma membrane which is devoid of other cell organelles (Carr, 1967; Daems & Brederoo, 1973). Microfilaments and microtubules are particularly prominent in pseudopods formed during the process of phagocytosis and adjacent to ingested particles as well as beneath the portion of the plasma membrane adhering to a surface (Reaven & Axline, 1973). The microfilaments may represent a contractile protein (Stossel & Hartwig, 1976).

Hirsch & Fedorko (1970) have described the differences between various developmental stages of mononuclear phagocytes. The Golgi apparatus and polyribosomes are more prominent in

promonocytes and stimulated macrophages as compared with monocytes and normal macrophages. This is correlated with a higher rate of synthesis and intracellular transport. Lysosomal granules are more numerous in macrophages particularly after in vitro or in vivo stimulation than in younger cells.

Alveolar macrophages contain residues of indigestible material such as carbon. Lipid droplets, endocytic vesicles, large vacuoles and convoluted extensions of the plasma membrane are particularly prominent in thioglycollate-induced peritoneal macrophages and in normal macrophages after 4 days of culture in 20% serum. The number of mitochondria, which reflects the rate of oxygen consumption, is larger in stimulated macrophages and alveolar macrophages than other types of mononuclear phagocytes.

Scanning electron microscopy reveals an irregular surface (Carr et al., 1969). It would be expected from transmission electron microscopy to find finger-like projections. These are seen along with flanges of protoplasm and 1-5 μm long ridges on trioleum-induced peritoneal macrophages which also vary greatly in size and shape. Normal peritoneal macrophages are more or less spherical and exhibit 0.5-2 μm long ridges which may represent finger-like processes that have fallen flat down on the cell surface as no such processes are seen. Carr & Carr (1970) followed the process of adherence and spreading on a surface using scanning electron microscopy. Initially the

cells stick to the surface and become deformed. After one hour finger-like projections can be seen extending from the cells and thin veils which seem to represent webs of cytoplasm creeping outwards between the projections. Fully extended cells are often markedly polarized with finger-like processes at the poles and a smoother upper surface than at the beginning.

6. BASAL METABOLISM

The following summary of the basal metabolism of unstimulated macrophages is meant to provide a background for the understanding of the metabolic changes that occur in macrophages in response to stimulation.

6.1 Uptake of nutrients.

Many cells are known to possess specific transport systems for nutrient substances in the plasma membrane and this has been shown also to be the case for mononuclear phagocytes. A transport system for glucose has been studied in peritoneal and alveolar macrophages using the undegradable glucose analogue, 2-deoxyglucose. This saturable system has the characteristics of carrier-mediated, facilitated diffusion with no transport against a concentration gradient (Mukkada & Bonventre, 1975; Gee et al., 1974). Uptake of glucose is independent of insulin (Gee et al., 1974) but is decreased by drugs that cause an increase in cyclic AMP (Khandwala & Gee, 1975).

Tsan & Berlin (1971a) studied the transport of amino acids in rabbit alveolar macrophages by measuring the uptake of radiolabelled amino acids employing a rapid sampling technique. They demonstrated a saturable transport system for lysin which could be inhibited to varying degrees by almost all natural amino acids. Leucine was one of the amino acids that competed for the lysine transport system but was in addition shown to be taken up by a separate transport system. The properties of these amino

acid transport systems in macrophages were shown to differ in several respects from those found in some other mammalian cells. The same workers have also demonstrated a transport system for the nucleotide adenosine (Tsan & Berlin, 1971b) which seems to be shared by uridine (Strauss & Berlin, 1973).

6.2 Fluxes and transport of electrolytes

Living cells maintain an electrolyte composition different from that of extracellular fluid. The concentration of K^+ is higher inside cells than outside, the reverse is true for Na^+ and Cl^- . Sodium and potassium are "pumped" across the plasma membrane by an energy-requiring process and diffusion of ions gives rise to a transmembrane electric potential with the inside of the cell negative relative to the outside (see Mountcastle, 1974). The intracellular concentration of Na^+ , K^+ and Cl^- in rabbit alveolar macrophages has been measured by Robin et al. (1971) and the values were found to be 83, 75 and 59 mEq/l respectively, and the ratios of concentrations extracellularly and intracellularly were 1.88, 0.08 and 0.51, in the same order. The concentration of Na^+ is intermediate between values quoted for erythrocytes from different species. It was found that Cl^- was in a rapid thermodynamic equilibrium whilst Na^+ had a rapid and a slow phase of efflux. The slow phase was found to be sensitive to ouabain which is a characteristic of the membrane adenosine triphosphatase (ATPase) responsible for ion pumping. The permeability for Na^+ was found to be high and this would

require a high activity of the membrane ATPase. Such an ATPase, stimulated by Na^+/K^+ and dependent on Mg^{++} , had been demonstrated previously in rabbit and sheep alveolar macrophages by Mustafa et al. (1969). Robin et al. (1971) calculated a membrane potential of -16 mV based on the measured ion concentration.

The membrane potential of mouse and guinea pig peritoneal macrophages and human blood monocytes was measured with microelectrodes by Gallin et al. (1975). The resting potential turned out to be -13.1 mV but in some of the preparations spontaneous hyperpolarizations to approximately -50 mV lasting 4-8 sec. occurred at an interval of 20-30 sec. The significance of this observation is unknown but these changes can be manipulated and it has been suggested that electrical phenomena might play a role in cell to cell communication between macrophages and lymphocytes (Rosenthal et al., 1976).

6.3 Bioenergetics.

Mononuclear phagocytes derive their energy from the oxidation of glucose (Oren et al., 1963). In peritoneal macrophages this occurs mainly by anaerobic glycolysis with the production of lactate but alveolar macrophages use oxidative phosphorylation as a source of energy (Oren et al., 1963; Karnovsky et al., 1970). The high rate of oxygen utilization in alveolar macrophages can be seen as an adaptation to an environment with a higher oxygen tension than the rest of the body. Recent studies by Simon et al. (1977) on the differences between alveolar and peritoneal macrophages with

respect to their bioenergetics have suggested that the level of enzymes involved in the utilization of oxygen is regulated by molecular oxygen itself. High levels of oxygen have been shown to lead to the formation of unstable metabolites that are noxious. One of these is the superoxide anion and it has been shown that alveolar macrophages possess a high activity of superoxide dismutase which disposes of this metabolite and this is rapidly stimulated further by an increase in oxygen tension (Rister & Baehner, 1977).

Macrophages keep a store of energy in the form of glycogen although the content in normal alveolar macrophages is only one tenth of that present in polymorphonuclear leucocytes (Khandwala & Gee, 1975) and glycogen granules are not very prominent morphologically in normal peritoneal macrophages (Daems & Brederoo, 1973). Caseinate-induced peritoneal macrophages, however, contain as much glycogen as polymorphonuclear leucocytes (Gudewicz & Filkins, 1976). These stores were rapidly depleted in glucose-free medium but could be maintained in medium containing either glucose or glycogen. Macrophages are obviously well equipped to obtain glucose from glycogen as they were shown to contain phosphorylase and in addition an acid α 1,4-glucosidase found in lysosomes.

6.4 Synthesis, storage and secretion.

Like other cells mononuclear phagocytes are constantly engaged in resynthesis and turnover of cell constituents such as the plasma membrane and this will be dealt with separately. In addition normal macrophages contribute to the overall metabolism of the body particularly the turnover of iron, production of bilirubin and plasma proteins and they may also play a role in lipid metabolism.

It is well known that macrophages, particularly in the spleen, take up effete red blood cells and destroy them (see Carr, 1973). Macrophages have been shown to break down haemoglobin to amino acids probably by means of lysosomal proteolytic enzymes (Ehrenreich & Cohn, 1968). It had been believed for a long time that bilirubin was formed mainly in the "reticuloendothelial system" and this was based on in vivo experiments where the reticuloendothelial system was blocked by agents such as thorotrast (Gottlieb, 1934a, b, c; Dumont et al., 1962). Pimstone et al. (1971) have shown that the enzyme haem-oxygenase which leads to the formation of bilirubin from haem can be induced in peritoneal and alveolar macrophages by in vivo exposure to methaemoglobin even though it can not be detected in macrophages from these sites from untreated animals. The highest activity of this enzyme is usually found in the spleen and it is also found in the liver and bone marrow. After

injecting damaged red blood cells containing radioactive iron into rats they were found to be taken up by the spleen and liver and most of the radioactive iron was released into the circulation within a few hours a small proportion being retained in the cellular iron store (Lipschitz et al., 1971). The fate of iron ingested by macrophages has been studied using iron-dextran which can be distinguished from ferritin on electron microscopy (Muir & Goldberg, 1961). The iron-dextran complex injected s.c. into mice was ingested into pinocytotic vacuoles. As early as 24h later ferritin granules could be seen in the cytoplasm and they subsequently accumulated in the vacuoles, gradually replacing the iron-dextran. Ferritin in vacuoles, the storage form of iron in the body, was found up to a year after injecting the iron-dextran. Macrophages can also take up iron bound to its transport protein transferrin for which they possess a specific receptor (MacSween & MacDonald, 1969; Wyllie, 1977). The degree of uptake varies with the iron saturation of transferrin (Wyllie, 1977). The affinity of the transferrin receptor also seems to vary according to the total iron stores of the animal. In iron-deficient animals the optimal saturation shifts from 80% to 40% and the uptake is lower in macrophages from iron-overloaded animals (MacSween & MacDonald, 1969). These results suggest a regulatory role for macrophages in the storage and transport of iron and availability of iron for synthesis (MacSween & MacDonald, 1969; Wyllie, 1977).

The transferrin that was taken up was not destroyed by the macrophages but released rapidly again in transferrin-free medium (Wyllie, 1977).

Macrophages are also known to be very active producers of transferrin as well as other plasma proteins, particularly complement components, as was studied in vitro by assaying proteins formed from labelled amino acids by Stecher & Thorbecke (1967). Macrophages produce the C2, C4, C5 and possible also C3 components of complement (Colten, 1976). Lysozyme is constantly synthesized and secreted by mononuclear phagocytes and the rate of production is not influenced by external stimuli including phagocytosis (Gordon et al., 1974a).

Lipid metabolism by macrophages has been studied particularly in relation to the pathogenesis of atherosclerosis as the lipid-laden macrophage is one of the features of the atheromatous plaque (reviewed by Day, 1964; see also Carr, 1973). It has been shown that macrophages can easily take up triglycerides (particularly if these are artificial), cholesterol and cholesterol esters as well as phospholipids. The uptake of lipoproteins seems to vary according to their composition. Fatty acids and triglycerides are oxidized and cholesterol esters hydrolysed. Macrophages can also synthesize cholesterol and fatty acids from 2-carbon fragments and form triglycerides, phospholipids and cholesterol esters. Ingested cholesterol can be shown to remain in the cells for up to 18 months (Day, 1964). The in vivo significance of this active lipid metabolism is not clear and the role of

macrophages in the pathogenesis of atherosclerosis has not been emphasized in more recent literature (see Frohlich, 1976).

Mononuclear phagocytes may also play a role in the metabolism of steroids. Berliner et al. (1964) have shown that adrenal macrophages hydroxylize carbon atom 17 of progesteron and thus produce more cortisol than parenchymal adrenal cells, the main product of which is corticosteron. Liver macrophages reduce the ketone in ring A of corticosteroids and give rise to products that have no longer the previous hormone properties, but steroids with a reduced ring A have been shown to have other properties such as pyrogenic, anaesthetic and hypocholesterolaemic (Nabors et al., 1967). Conjugation of steroids to water soluble compounds occurred only in the parenchymal cells of the liver.

7. THE MACROPHAGE PLASMA MEMBRANE

The present study concerns itself with two functions of the macrophage plasma membrane; recognition and signal transmission. It is, therefore, relevant to consider in some detail the structure and metabolism of plasma membranes with special reference to the macrophage plasma membrane.

7.1 The mammalian plasma membrane.

The cell membrane is the external boundary of the cell but it also forms the link between the cell and its environment, it is concerned with cellular recognition phenomena, receives signals and transmits them to the cytoplasm.

7.1.1. Experimental methods. The mammalian plasma membrane has been the subject of very active research in recent years. The cell membranes of erythrocytes have been widely used in these studies. They have the advantage that both sides of the membrane can be explored as the erythrocyte ghosts can be turned inside out or allowed to incorporate substances such as enzymes and then resealed. Erythrocytes, however, are somewhat different from ordinary nucleated cells which have been more used in functional studies of the plasma membrane. The techniques employed include extraction and chemical analysis of components, physicochemical techniques, treatment with specific enzymes and labelling of individual components of intact membranes. The

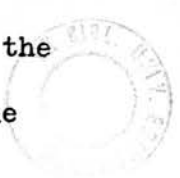
physicochemical techniques can involve direct measurements by e.g. x-ray diffraction, nuclear magnetic resonance spectroscopy or differential scanning calorimetry, or may require the insertion of a probe such as fluorescent or spin-label probes. In this way direct information can be gained about lipid and protein packing, fluidity and microviscosity, lateral diffusion and transmembrane rotation, and by comparison with the physicochemical parameters of artificial membranes with known structure the likely structure of biological membranes can be inferred (see review by Nicolson et al., 1977). Enzyme treatment or labelling of intact membranes with e.g. antibodies, lectins and fluorescent substances show which components are expressed on membrane surfaces (Singer & Nicolson, 1972; Bretscher, 1973; Steck et al., 1971). A great deal has been learnt about the composition and overall structure of the plasma membrane, but the finer details of surface architecture and how these may be controlled by cytoplasmic elements are still being actively investigated and speculated upon.

7.1.2. Composition and structure of the plasma membrane.

The plasma membrane is composed mainly of proteins and lipids at a weight ratio of about 1.5-4 (Singer & Nicolson, 1972), about 10% or less of the mass is accounted for by carbohydrates (Bretscher, 1973). Phospholipids constitute the majority of the lipids but there are also large amounts of cholesterol (Bretscher,

1973). The carbohydrate is found as part of glycoproteins and glycolipids (Bretscher, 1973).

The fluid mosaic model of the plasma membrane was introduced by Singer & Nicolson (1972) and is now generally accepted (see Gomperts, 1977). This model was arrived at by considering how phospholipids and proteins could form a thermodynamically stable system. The proteins are envisaged as floating in a semiliquid bilayer of phospholipids. In this phospholipid bilayer the polar headgroups of the phospholipids are exposed to water at the outer and inner surface and the fatty acid chains meet in the middle. Membrane proteins can be divided into peripheral proteins and integral proteins. The former can be removed intact by relatively mild treatment, such as increasing the salt concentration or adding chelating agents, without disturbing the integrity of the membrane. The integral proteins, which constitute over 70% of the membrane protein, can only be dissociated by more drastic methods such as treatment with detergents, organic solvents or denaturants; they often take with them bound lipid but, if completely free of lipid, they are highly insoluble in water (Singer & Nicolson, 1972). These proteins penetrate the lipid bilayer to varying depths, some are completely embedded, others have portions extending beyond the inner or outer or both bilayer surfaces (Singer & Nicolson, 1972; Nicolson & Poste, 1976). The embedded portions are hydrophobic and therefore in thermodynamic equilibrium with the non-polar environment of the phospholipid bilayer, whilst the



polar ends of the proteins, containing ionic amino acids, are exposed to the aqueous environment of extracellular fluid or cytoplasm.

Steck et al. (1971) found 6 major proteins and 3 glycoproteins on sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis of erythrocyte membrane proteins, numerous additional faint bands can be seen, representing proteins present in too low quantities to be analyzed (Bretscher, 1973).

The main phospholipids of the membrane are phosphatidylcholine, sphingomyelin, phosphatidylethanolamine and phosphatidylserine, with phosphatidylcholine and phosphatidylethanolamine being most abundant (Bretscher, 1973). Phospholipase digestion of intact erythrocytes and erythrocyte ghosts has demonstrated that the outer leaflet is mainly composed of the choline phospholipids (phosphatidylcholine and sphingomyelin), in varying proportions according to species, whereas the amino phospholipids (phosphatidylethanolamine and phosphatidylserine), are found almost exclusively in the cytoplasmic half (Zwaal et al., 1975). These results are supported by experiments using different techniques such as chemical labelling (Bretscher, 1973). Neutral lipids also are probably not distributed evenly, the outer layer appears to contain about twice as much cholesterol as the inner leaflet (Bretscher, 1973). The membrane lipids exhibit free and quite rapid lateral movement in the plane of the membrane but trans-membrane "flip-flop" movements are rare (Bretscher, 1973; Nicolson et al., 1977).

The carbohydrate of glycoproteins is always exposed at the external surface and membrane glycolipids seem to be orientated in the same manner (Bretscher, 1973; Nicolson & Poste, 1976). The most commonly identified sugars include galactose, glucose, their amino- and N-acetylated derivatives, and sialic acids, fucose and mannose. Relative amounts vary considerably between cell types and species (Winzler, 1970; Fujita & Cleve, 1975; Snary *et al.*, 1976). In addition to membrane glycoproteins and glycolipids, mucopolysaccharides (glycosaminoglycans) are sometimes found associated with the external cell surface. These may, perhaps in conjunction with carbohydrate residues of the glycoproteins and glycolipids, form what has been called "cell coat" or "glycocalyx" and can be seen on electronmicrographs (Cook & Stoddard, 1973; Luft, 1977).

7.1.3. Functions of plasma membrane molecules.

Integral proteins can be shown to be in close contact with membrane lipids and may rely on specific lipids for their final structure and function as enzymes and transport molecules (Singer & Nicolson, 1972; Bretscher, 1973; Nicolson & Poste, 1976; Coleman & Bramley, 1975; Meister, 1976). The glycoproteins and glycolipids function as receptors for hormones and pharmacologically active agents (Cuatrecasas, 1974; Révész & Greaves, 1975) as well as lectins (Sharon & Lis, 1972) and

viruses (Fenner & White, 1970) and they can be surface antigens (Singer & Nicolson, 1972). The cell surface is negatively charged as can be demonstrated by electrophoresis of whole cells or binding of positively charged granules. This negative charge is carried mainly on sialic acid residues (Eylar et al., 1962; Weiss et al., 1972).

7.1.4. Lateral mobility of plasma membrane constituents.

The fluid mosaic model of the plasma membrane predicts that proteins will be randomly distributed in the lipid bilayer and be undergoing random lateral motion. This seems largely to be the case although the lateral motility of integral membrane proteins is lower than that of the lipids (Nicolson et al., 1977). There are, however, indications that certain proteins on highly specialized membranes such as at cell junctions are non-randomly distributed. In some cases such organized patterns may be controlled in the plane of the plasma membrane, "cis-control" (Nicolson et al., 1977). This could involve non-covalent forces acting on the hydrophilic zone or lateral phase separation within the lipid bilayer. There is also the possibility of trans-membrane control and this has been implicated to explain the restricted mobility observed for certain membrane proteins. Considerable evidence exists to show that the membrane-associated cytoskeletal elements, microfilaments and microtubules, control membrane movement and lateral motility of some proteins within the membrane. This evidence comes largely from studies on the

phenomenon of capping by surface ligands, such as lectins and antibodies, and the effect thereon of agents such as cytochalasin B, that disrupts microfilaments, and colchicine that depolarizes microtubules. Capping is an energy-dependent process and results in the coalescence of a surface protein at one pole of the cell when it has been exposed to multivalent ligands and follows aggregation of the surface protein into clusters and patches. Some of the studies employing cytochalasin B and colchicine have yielded conflicting results but the most common pattern is some inhibition of capping by cytochalasin B, no effect or enhancement by colchicine, and total block by colchicine and cytochalasin B together (Nicolson & Poste, 1976; Nicolson et al., 1977). In very simple terms the results have been interpreted to imply an anchoring function for microtubules that restricts mobility of membrane components to which they are "linked" whilst microfilaments function as contractile elements influencing movement of membrane components as well as effecting cellular locomotion and changes in cell shape (Nicolson et al., 1977).

7.1.5. Current models of cell surface architecture.

Two recent models of cell surface architecture and its trans-membrane control by Loor (1976) and Nicolson et al. (1977) are based on the theory on the function of microtubules and microfilaments stated above. In Loor's model microfilaments, composed of actin, extend into microvilli, α -actinin is depicted

at the tip of a microvillus and myosin is shown intertwined with the actin filaments. Microtubules run parallel to the membrane surface without extending into the microvillus, and they are seen crossing microfilaments. The plasma membrane contains peripheral and integral proteins. It is impossible because of size for single membrane proteins to be linked to several microfilaments or microtubules, as had been postulated before. Loor therefore shows the linkage of several plasma membrane proteins to single microfilaments and microtubules by means of α -actinin or cross-bridges between microtubules. The model of Nicolson et al. (1977) is essentially similar albeit more elaborate. Mucopolysaccharides are shown bound to the external portions of integral membrane glycoproteins. Peripheral proteins are present on the inner side of the membrane and are shown to interact with the membrane-associated cytoskeletal elements. Microfilaments, containing actin and tropomyosin, lie parallel to the cell surface as well as extending into microvilli. This is supported by recent electronmicrographs using immunofluorescence (Lazarides, 1975). Microfilaments appear to be crosslinked by α -actinin and adjacent arrays linked by myosin. Various bridge molecules of unknown identity are depicted linking microfilaments to the membrane and forming links between microtubules. As microfilaments and microtubules have opposing and co-ordinated roles in controlling movement and distribution of membrane proteins they must be

connected to each other or to similar structures on the inside of the membrane. The nature and identity of the linkage molecules and linked trans-membrane components is largely unknown but in erythrocytes the peripheral protein spectrin and the trans-membrane protein glycophorin seem to fulfil such functions (Nicolson, 1973; Nicolson et al., 1977).

Recent morphological evidence obtained with immunofluorescent stains supports the concept of a link between cytoskeletal elements and membrane components. Sundquist & Ehrnst (1976) showed that redistribution of actin into blebs and bulges caused by cytochalasin B leads to the accumulation of certain surface antigens but not others in corresponding areas. Ash & Singer (1976) demonstrated linear organization of concanavalin A receptors superimposed on myosin filaments. Actin and myosin were identified by Bourgignon and Singer (1977) in patches and caps directly corresponding to surface patches and caps of concanavalin A receptors. These authors put forward the hypothesis that actin behaves as a peripheral protein and is linked to a putative integral protein X which binds selectively to surface receptors when they are aggregated into patches by multivalent ligands. The patches would then be gathered into a cap by a sliding filament mechanism.

7.1.6. Control of microfilament and microtubule

functions. Little is known about the control of microfilament and microtubule function. They are probably both influenced by nucleotides and divalent cations. Calcium ions promote the depolymerization of microtubules (Kirschner et al., 1974) and Ca^{++} is well known to control actomyosin action in muscles. Bound guanosine triphosphate (GTP) is required for the assembly of microtubules and during the process GTP is hydrolysed which probably promotes depolymerization. Adenosine triphosphate (ATP) binding and hydrolysis may have a similar function in actin polymerization (Weisenberg & Deery, 1976). Cyclic adenosine monophosphate (cAMP) inhibits lymphocyte motility following treatment with anti-immunoglobulins whilst cyclic guanosine monophosphate (cGMP) has a stimulatory effect (Schreiner & Unanue, 1975). As colchicine prevented the effect of cAMP the authors concluded that cAMP might have a rapid polymerizing effect on microtubules. The results of Oliver et al. (1975) appear somewhat contradictory, they found that cGMP could block capping in polymorphonuclear leucocytes from beige (Chediak-Higashi) mice or colchicine-treated normal polymorphonuclears thus suggesting that cGMP promotes microtubule assembly.

7.1.7. Turnover of plasma membrane constituents.

Membrane proteins are constantly being shed and degraded at different rates and renewed (Cone et al., 1971). It is not known

whether this renewal occurs by independent insertion of separate molecules into the membrane or by an assembly line process. In the latter process polypeptides are envisaged to be synthesized on the rough endoplasmic reticulum and transferred to the smooth endoplasmic reticulum and then to the Golgi apparatus where they are formed into vesicles which migrate to the plasma membrane to fuse with it. This latter hypothesis was favoured in the review by Nicolson et al. (1977). The rate of turnover seemed to vary considerably between cell types with recorded half-lives ranging from a few hours to 18 days or 10-30% of cell generation time (Nicolson et al., 1977). Lymphocytes seemed to possess a reserve pool of surface components which was gradually used up in the presence of metabolic inhibitors (Cone et al., 1971). Carbohydrates on glycoproteins or glycolipids can be modified on the cell surface by glycosyltransferases (Roseman, 1970; LaMont et al., 1977). The plasma membrane can not synthesize phospholipids and according to the assembly line theory they are synthesized intracellularly and transferred to the membrane as ready made vesicles with membrane proteins. The existence of specific phospholipid exchange proteins which transport phospholipids between cellular membranes has recently been demonstrated; these exchange proteins may contribute to the building of the plasma membrane as well as the repair following damage by phospholipases (Kader, 1977).

7.1.8. Variations in expression of cell surface components. Finally it should be mentioned briefly that the expression of certain cell surface components, particularly carbohydrates, can be altered during the different phases of cell growth and upon malignant transformation. Thus malignant cells as well as normal cells in mitosis can be agglutinated by lectins whilst normal cells in interphase bind lectins but are not agglutinated. This seems to be related to altered expression and mobility of membrane glycoproteins perhaps sometimes linked to defects in the cytoskeleton (see reviews by Sharon & Lis, 1972; Gahmberg, 1977). A cell membrane glycoprotein, called fibronectin, is absent from transformed cells and present only in very small amount in mitotic normal cells. In normal cells synthesis of this protein becomes accelerated in the G_1 -phase of the cell cycle when it reappears on the surface. These findings suggest a role for this protein in the regulation of cell growth (Gahmberg, 1977). Cifone & Defendi (1974) have described the cyclic expression of macrophage growth factor on the surface of L-cells during S-phase, the factor being released into the medium after metaphase. Some of the variations in surface carbohydrates may be caused by variations or aberrations in glycosyltransferase activity (LaMont et al., 1977).

7.2 Composition and turnover of the macrophage plasma membrane.

7.2.1. Composition of the macrophage plasma membrane.

The chemical composition of the plasma membrane of alveolar macrophages from rabbits stimulated with BCG was analyzed by Nachman et al. (1971a). They used two methods for separation of plasma membrane; separation on sucrose gradients of homogenates of glutaraldehyde-fixed cells or homogenates of unfixed cells following ingestion of latex particles. The latter method yields phagolysosomal membranes. It was found that 46% of the membrane mass was protein, 41% was lipid, 8% were accounted for by carbohydrate, 3% by RNA (which is fairly high) and 0.2% by DNA. Amino acid analysis gave results similar to those obtained for other cells although there was a relatively high content of aspartic and glutamic acids. Seven to nine major proteins were defined on SDS polyacrylamide gel electrophoresis, with molecular weights of 7×10^4 to 14×10^4 , only one showed a positive reaction for carbohydrate but two contained lipid. Phagolysosomal membranes contained the same proteins as plasma membranes prepared from non-phagocytosing cells but in slightly different proportions. Membrane-bound enzymes have been found in plasma membranes of macrophages as in membranes of other cells. These include 5'-nucleotidase, adenosine triphosphatase, p-nitrophenylphosphatase (Karnovsky et al., 1975a) and alkaline phosphodiesterase (Wang et al., 1976a). The lipid composition

of alveolar macrophages, whole cells and phagocytic vesicles, has been studied by Mason et al. (1972). As expected phosphatidylcholine, sphingomyelin and phosphatidylethanolamine were found to be the major phospholipids but an unusual lipid, lyso(bis)phosphatidic acid which has a free phosphate group, was present in similar amounts. Phosphatidylserine and phosphatidylinositol were found in smaller quantities as well as very small amounts of lysolecithin and polyglycerolphosphate. The relative amounts of sphingomyelin and lyso(bis)phosphatidic acid were higher and that of phosphatidylcholine lower in phagocytic vesicles when compared with whole cells. The phagocytic vesicles also contained a higher proportion of cholesterol and polyunsaturated fatty acids. The differences in relative composition between whole cells and phagocytic vesicles was interpreted by the authors as a selective inclusion of membrane portions rich in particular lipids but it could also partly be caused by the action of lysosomal phospholipases (Franson & Waite, 1973) as there was also an increase in lysolecithin. Hanada et al. (1978) have recently investigated the glycolipids from rat macrophages and proposed the structure for the major glycolipid, called glycolipid X. This glycolipid has high bloodgroup B activity and is unusual in containing galactosamine but no N-acetylglucosamine which is normally found in ABO-reactive glycolipids. The other sugars present are three galactose molecules, one fucose and one glucose.

A delicate and very loosely attached "cell coat" composed of protein was described on Kupffer cells by Emeis & Wisse (1975). This coat was very easily removed by gentle washing and was absent from pseudopods. If this coat is genuine it is unusual for being a protein rather than a carbohydrate. Dvorak et al. (1972) have described a carbohydrate "cell coat" or "glycocalyx", more akin to that described on other cells, on guinea pig peritoneal macrophages. This was seen mainly as patches and a thin layer on blunt pseudopods but not on long villi. It was decreased in quantity following exposure to migration inhibition factor particularly at points of cell contact.

7.2.2. Turnover of the macrophage plasma membrane.

The turnover of macrophage plasma membrane proteins was studied by Nachman et al. (1971b) by following the incorporation of labelled leucine into the different membrane proteins which were separated in SDS polyacrylamide gel electrophoresis. There was little uptake during the first 45 minutes but then the radioactivity of membrane proteins increased rapidly until 90 minutes of incubation when it started to decrease slightly again. The rate of uptake into different proteins was not uniform, three of the main proteins showed peak uptake after 90 minutes but in two of them the uptake was higher after 150 minutes. This seems to indicate different rates of synthesis for individual membrane proteins. The half-life of plasma membrane proteins

was measured by pulse-chasing with cold leucine following a 90 minute incubation with labelled leucine and was found to be 7-8 hours. The label appeared to be lost at a fairly similar rate from most of the major proteins which was attributed to the bulk removal of membrane by pinocytosis. If this is the case the proteins must be degraded quite rapidly following internalization as the disappearance of label from whole cell protein was not much slower than the rate of loss from membrane protein. An alternative way of disappearance of labelled membrane protein would be by shedding as has been shown for lymphocytes (Cone et al., 1971). Labelled glucosamine was incorporated into two bands and a high proportion remained at the origin of the SDS polyacrylamide gel where the only positive reaction for carbohydrate had been found. There was also marked uptake of (^3H)-choline mainly into phosphatidylcholine but also found in lysolecithin. The two proteins that stained for lipid also incorporated the labelled choline (Nachman et al., 1971a). Cohn & Benson (1965b) followed by autoradiography the uptake of two of the radioactive substances used in the studies just described, (^3H)-leucine and (^3H)-choline. Both substances were first seen in the peripheral cytoplasm, and then after one hour in perinuclear vesicles. There was no direct uptake into the plasma membrane. This makes it likely that plasma membrane synthesis takes place intracellularly.

Werb & Cohn (1971a and b) have studied the metabolism of cholesterol in mouse peritoneal macrophages. More than 95% of cellular cholesterol was associated with cellular membranes mainly the plasma membrane and lysosomes. Cholesterol was rapidly taken up from serum-containing medium, less than 0.1% of cell cholesterol was synthesized from labelled acetate. One third of the total cholesterol content was exchanged during the first hour which is considerably faster than the turnover rate of proteins found by Nachman et al. (1971b). The curve describing the exchange of labelled cholesterol revealed a rapid and a slow phase indicating the presence of two compartments, one exchanging cholesterol rapidly constituting 60-70% of the total cholesterol pool the other having a slower rate of exchange (Werb & Cohn, 1971a). The slowly exchanging compartment was shown to be lysosomal membranes and was increased in relative size following pinocytosis or phagocytosis (Werb & Cohn, 1971b). The exchange was independent of uptake by endocytosis but required a trypsin-sensitive membrane protein. Following trypsin-treatment the return to normal exchange rate took 7 hours and required protein synthesis and the presence of serum (Werb & Cohn, 1971b). These experiments have thus also provided some information on the rate of synthesis of membrane proteins. Trypsin also removes membrane components involved in the pinocytosis of protein (Lagunoff, 1971) and phagocytosis of aldehyde-treated red blood cells (Werb, 1975)

and the recovery takes 4-6 hours and 5-8 hours respectively. By contrast Ilgen and Burkholder (1974) found that a trypsin-sensitive C4-determinant on macrophages was regenerated within a few minutes but as C4 is secreted constantly by macrophages this may behave differently from ordinary membrane proteins.

Several years ago Karnovsky and co-workers measured the incorporation of labelled inorganic phosphate into phospholipids (Graham et al., 1967; Karnovsky et al., 1970). Phosphatidyl-inositol and phosphatidylserine (combined) and phosphatidylcholine showed considerably higher uptake than phosphatidylethanolamine, phosphatidic acid or sphingomyelin. This is not correlated with the relative proportions of these lipids in the plasma membrane. Phagocytosis and digitonin caused a marked increase in labelling only of the phosphatidylinositol-phosphatidylserine fraction. The active and variable turnover of the phosphate of certain membrane phospholipids is probably not related to the general turnover and synthesis of membrane constituents as will be discussed later. Recently Wang et al. (1976b) discovered the presence of the enzyme cytidine diphosphocholine phosphotransferase in the plasma membrane as well as the endoplasmic reticulum of rabbit alveolar macrophages. This enzyme is only found in the endoplasmic reticulum in other cells and is involved in the final step of the de novo synthesis of phosphatidylcholine, one of the major plasma membrane phospholipids. The macrophage plasma membrane is thus capable of synthesizing phosphatidylcholine from diglyceride

and cytidinediphosphocholine in situ. Another enzyme, monoacyl phospholipid transferase, which is concerned with resynthesis of phospholipids from breakdown products of phospholipase activity, was found only in the endoplasmic reticulum.

7.2.3. Renewal of plasma membrane following phagocytosis. Macrophages are actively pinocytic and phagocytic cells and can thus internalize a large proportion of their surface membrane which then has to be renewed. Steinmann et al. (1976) have calculated that macrophages internalize the equivalent of their surface every 33 minutes through pinocytosis; the rate for L-cells was 4 times slower. There may be some recycling of pinocytosed cell membrane back to the surface (Steinman et al., 1976) but much of the renewal following phagocytosis of particles seems to occur by synthesis of new membrane as is indicated by a lag period of 5-6 hours before reconstitution of the plasma membrane and requirement for protein synthesis and cholesterol. Thus Werb & Cohn (1972) found that phagocytosis, pinocytosis, cell spreading and cholesterol uptake were depressed for about 5 hours after ingestion of particles and the presence of cholesterol was required for recovery. The membrane content of phospholipids and cholesterol started to increase after a lag period of about 4-8 hours and the net final increase was linearly related to the number of latex particles ingested. This increase in membrane

lipids was dependent on protein synthesis and the presence of serum.

The level of the membrane enzyme $5'$ -nucleotidase was markedly decreased following phagocytosis and internalized enzyme was degraded in the lysosomes. The activity of $5'$ -nucleotidase in the membrane started to return to normal after 6 hours, reaching normal levels after 10 hours, and this again depended on protein synthesis and the presence of serum and cholesterol. The enzyme $5'$ -nucleotidase may, however, not be a very suitable marker to use as indicator of overall renewal of membrane proteins. It might seem that the level of this enzyme in the membrane is determined to some extent by factors other than the integrity of the membrane. Some workers have failed to detect this enzyme in the plasma membranes of activated alveolar macrophages (Wang et al., 1976a) and it is very markedly decreased in peritoneal macrophages elicited by caseinate or peptone (Karnovsky et al., 1975a). The activity is also lowered in peritoneal macrophages from mice injected with lipopolysaccharide, and this has been shown to be due to increased breakdown. In contrast the activity of this enzyme increases during in vitro culture (Edelson & Cohn, 1976). The half time of its synthesis was found to be 13.6 hours.

Schmidt & Douglas (1972) found that phagocytosis of latex or aldehyde-treated red blood cells caused a very marked decrease in IgG-receptor activity which recovered almost fully within 4-6 hours. Phagocytosis also causes reduced binding of concanavalin A to macrophages and this has come back to 90% of normal after $4\frac{1}{2}$ hours with full recovery after 8 hours (Lutton, 1973). Skutelsky & Hardy (1976) noted patchy regeneration of anionic sites on the macrophage membrane after these had been removed by cationic ferritin. Full regeneration occurred within 3 hours. The pattern of regeneration was said to argue against regeneration of plasma membrane in bulk but would still seem to be compatible with the assembly line theory of membrane regeneration as described above.

The study of membrane renewal following phagocytosis is further complicated by the observation that internalization of membrane components during phagocytosis does not seem to occur completely at random. Thus Tsan & Berlin (1971b) found that membrane transport systems for amino acids and nucleotides in polymorphonuclear leucocytes and macrophages were completely unaffected by phagocytosis. Subsequently it was shown that in polymorphonuclear leucocytes the transport molecules were no longer retained at the surface if phagocytosis occurred in the presence of colchicine (Ukena & Berlin, 1972) which also prevented the loss of concanavalin A binding sites normally

observed after phagocytosis (Oliver et al., 1974). Colchicine, which had no effect on phagocytosis itself, thus prevented the segregation of membrane proteins during phagocytosis suggesting that this is normally brought about by cytoskeletal elements. The studies with colchicine have not been repeated with macrophages.

The experiments of Gordon & Cohn (1970, 1971 and 1973) with heterokaryons of macrophages and melanocytes or L-cells have provided evidence for random lateral movement of macrophage membrane proteins in the absence of phagocytosis. A macrophage ATPase was found in discrete regions shortly after fusion with melanocytes and then distributed more diffusely before it disappeared after about 12 hours (Gordon & Cohn, 1970). The IgG-receptor followed a similar pattern and their disappearance could be attributed to masking by a trypsin-sensitive protein of melanocyte origin (Gordon & Cohn, 1971). Heterokaryons of macrophages and L-cells expressed surface antigens of both parents (Gordon & Cohn, 1973).

7.3 Sugars and antigens on the macrophage surface.

The expression of membrane components on the cell surface can be investigated with substances that are known to bind specifically to certain molecules or molecular configurations such as lectins and antibodies.

7.3.1. Sugars on the macrophage surface. Wollweber & Fritsch (1975) demonstrated binding of concanavalin A (ConA), ricinus communis agglutinin (RCA) and wheat germ agglutinin (WGA) to mouse peritoneal macrophages. These lectins are specific for glucose and mannose (ConA), N-acetylglucosamine (RCA) and galactose (WGA). These results are supported by the findings of Goldman et al. (1976) who demonstrated binding of these lectins and others with similar specificities and also noted binding of Lotus tetragonolobus lectins which are specific for fucose.

7.3.2. Surface antigens of macrophages. Macrophages have been shown to share antigens with other cells but they also possess surface antigens that are not found on other cells. Anti-mouse-macrophage serum can be raised in rabbits. When such an antiserum was subjected to sequential absorption with mouse erythrocytes, lymphocytes and granulocytes the presence of three antigens on the macrophage was demonstrated (Tiller et al., 1973). One of these was shared with lymphocytes, a second with granulocytes and fibroblasts and a third appeared to be confined to macrophages. The absorbed antiserum stained only 20% of alveolar macrophages. Stinnett et al. (1976) have isolated a membrane protein from mouse peritoneal macrophages by immunoprecipitation with extensively absorbed anti-macrophage serum. This protein, which the authors call

mouse specific macrophage antigen (MSMA) has a molecular weight of 8.3×10^4 daltons. This antigen was not found on non-adherent peritoneal exudate cells, bone marrow cells or red blood cells from the mouse, but was found on normal and activated peritoneal exudate macrophages, allogeneic peritoneal exudate macrophages and a macrophage cell line, P388/D. There was cross-reactivity with peritoneal exudate macrophages from rat and hamster but not guinea pig. Mouse macrophages have also been shown to express an allogeneic marker, called Mph-1 (Archer, 1975). This marker maps on chromosome 7 not far from some minor histocompatibility antigens. It shows co-dominant inheritance like H-2 and Thy-1. There are two alleles; Mph 1.1, found in only two strains of mice (F/St and I/StDa), and Mph 1.2, found in the other strains tested, including C3H/He, CBA/Ca, AKR and others.

Macrophages express antigens of the K and D regions of the major histocompatibility complex (H-2) in the mouse and recently antigens linked to the immune response genes (Ir) of the H-2 complex, Ia-antigens, have also been demonstrated (Hämmerling et al., 1975). Ia-antigens have also been shown to be expressed on guinea pig macrophages (Schwartz et al., 1976a and b). HLA-D antigens, which are believed to be the equivalents of Ia-antigens in the mouse, have been reported on human peripheral blood monocytes (Hirschberg et al., 1976; Albrechtsen, 1977). There are indications that the Ia-antigens on guinea

pig macrophages and Ia-like determinants on human monocytes may be somewhat different from those expressed on B-cells which are used to produce and test the specific antisera (Schwartz et al., 1976a; Moraes et al., 1977). The presence of Ia-antigens on macrophages is of special interest with regard to their role in the initiation of the immune response and the requirement of histocompatibility for the interaction of T-cells and macrophages (Rosenthal et al., 1976) as well as the possibility that immune response gene function may be expressed at the level of antigen presentation on the macrophage (Rosenthal et al., 1977). In addition Erb & Feldmann (1975a) have demonstrated a requirement for identity at the I-A subregion of the Ir region for macrophage-dependent production of helper T-cells (from mice) in vitro. It has become clear that only a small proportion of mouse peritoneal macrophages synthesize and express Ia-antigens (Schwartz et al., 1976b). This has recently been investigated further and it was found that whilst 85% of splenic macrophages express Ia-antigens less than 10% of peritoneal macrophages do so (Cowing et al., 1978). The sub-region expressed is mainly I-A, with a minor population bearing I-C determinants. The splenic macrophages were found to lose their Ia-antigens during 4 days of in vitro culture in the presence of 20% of heterologous serum at which time they showed extensive spreading. Niederhuber & Schreffer (1977)

have studied the effect of anti-Ia sera on macrophage function in the primary antibody response in vitro. Pretreatment of the macrophages with anti-Ia sera was found to block their ability to promote this response and this was most marked with antisera directed against the J sub-region of the Ir region. The sub-region specificity seems to be in disagreement with the results of Erb & Feldmann (1975a) and the predominant expression of subregion A on macrophages (Cowing et al., 1978), see above. The function assayed by Niederhofer & Schreffer (1977), IgM production, is different from the more frequently measured IgG-response, this might in part account for the discrepancy. In the guinea pig the Ia-antigen-positive macrophages also constitute only a minority among peritoneal macrophages. After these macrophages had been killed by anti-Ia serum and complement the remaining macrophages could no longer induce T-cell responses to protein antigens or a mixed lymphocyte reaction (Yamashita & Shevach, 1977). These findings lend support to the concept of functional heterogeneity of macrophages. This could be based on fully differentiated subpopulations or different stages of maturation as would be suggested by the loss of Ia-antigen from splenic macrophages during culture.

8. RECOGNITION

The present study deals to a large extent with non-specific recognition by macrophages. The following chapter reviews some general aspects of biological recognition, both specific and non-specific, as well as the reported specific receptors on macrophages and observations on non-specific recognition by macrophages.

8.1 Biological recognition.

Cellular recognition can be said in broad terms to describe that property of living cells to respond in a predictable way when chemical substances, living or dead particles or surfaces come into contact with their surface. This can be reflected in responses such as surface binding, internalization, cell movement, aggregation, synthesis, secretion and cell division. Recognition of molecules is often mediated by specific receptor molecules in the plasma membrane and recognition of particles can occur with the help of recognition substances that react with chemical specificity with molecules on the target and are in turn recognized by the cells through their specific plasma membrane receptors. In other cases recognition of particles, cells or surfaces seems to occur without the involvement of such recognition molecules.

8.1.1. Specific cellular receptors. The concept of specific cellular receptors was developed to explain the mode of action of drugs and hormones (see Ariëns & Beld, 1977). According to this theory, originally introduced by Langley (1905), a drug can only exert its biological effect after binding to a "receptive substance" in the target tissue. Recent experimental work has provided evidence for the existence of such receptors as molecular entities. Thus radiolabelled hormones can be shown to bind in a specific way and with high affinity to cell homogenates or isolated membranes of target tissues and the surface of target cells. The binding has been shown to be correlated with the biological effect (Cuatrecasas, 1974; Catt & Dufau, 1977). These receptors can be studied by biochemical methods and the reaction kinetics, affinity and specificity of binding and the number of receptor on the cell surface can be established (Cuatrecasas, 1974). The nature of receptor molecules can be investigated by exposing whole cells or isolated membranes to enzymes. This approach has demonstrated that receptors for different hormones vary in their susceptibility to various enzymes which supports the concept of specific receptors. Thus the receptor for insulin on fat cells is destroyed by trypsin whilst the receptors for adrenalin and ACTH on the same cells are not affected (Kono, 1969a) and phospholipase treatment of liver cells causes decreased binding of glucagon but unmasks hidden receptors

for insulin on fat cells (Rodbell et al., 1971; Cuatrecasas, 1974). From studies of this kind the receptor for insulin appears to be a glycoprotein and galactose groups seem to be directly involved in the binding of the hormone as judged by the great decrease in binding caused by treatment of the cells with β -galactosidase (Cuatrecasas, 1974).

8.1.2. The nature of receptor molecules.

Receptor molecules can be isolated from plasma membranes by solubilization in a non-ionic detergent (such as Triton X-100) followed by protein fractionation and purification by affinity chromatography on gel columns coated with the substance binding to the receptor. Several receptors have now been isolated in this way including the nicotinic receptor for acetylcholine (Biesecker, 1973; Ong & Brady, 1974), receptors for various peptide hormones (Cuatrecasas, 1974; Giorgio et al., 1974; Catt & Dufau, 1977) and a receptor on liver cells for asialated glycoproteins (Ashwell & Morell, 1974). These receptor molecules are proteins with a molecular weight of 200,000 to 300,000 (Biesecker, 1973; Cuatrecasas, 1974; Catt & Dufau, 1977) and they may be built up from smaller subunits (Biesecker, 1973; Ong & Brady, 1974) sometimes as dimers (Catt & Dufau, 1977). Many of the receptor proteins have been shown to contain carbohydrate (Catt & Dufau, 1977) and the insulin receptor binds to the lectins Concanavalin A and wheat germ haemagglutinin (Cuatrecasas, 1974). Some of these receptors may be associated

with or affected by membrane phospholipids as suggested by the effect of phospholipases on their activity as mentioned above. The highly specific surface receptor for antigen on B lymphocytes is probably identical with membrane immunoglobulin (see Golub, 1977) and thus another example of a receptor that is a membrane glycoprotein. Glycolipids can also function as specific receptors for pharmacologically and physiologically active agents as has been demonstrated for the receptor for cholera toxin (Révész & Greaves, 1975). In some cases the binding of a drug does not require a receptor of high structural specificity. Gaseous anaesthetics, for example, accumulate in the lipid bilayer of the plasma membrane by virtue of their lipophilicity and thereby cause a change in membrane properties (see Ariëns & Beld, 1977).

8.1.3. Mechanisms of binding to receptors. Little is known about the precise physicochemical nature of binding to a receptor. Binding is often influenced by ions, thus the binding of glucagon involves divalent cations (Rodbell et al., 1971) and an increase in the concentration of Na^+ promotes binding of angiotensin II to adrenal cells (Glossmann et al., 1974). Sodium is required for the binding of agonists but not antagonists to the opiate receptor and this has led to the suggestion that this receptor exists in two states dependent

on the presence or absence of Na^+ (Simantov & Snyder, 1977). The binding may sometimes involve hydrophobic interactions perhaps aided by hydrogen bonds; this has been suggested as the mechanism of binding of insulin. In other cases (such as the binding of ACTH and angiotensin II) electrostatic effects of ionic amino acids on the hormones appear to be important (see Catt & Dufau, 1977). The binding of human chorionic gonadotropin appears to be mediated by its sugar residues (Bahl, 1977).

8.1.4. Lateral motility of receptors in the plasma membrane. Receptors for hormones and drugs can move laterally within the plasma membrane as expected from the fluid mosaic membrane model. This may be essential for the biological consequences of hormone binding as suggested in the mobile receptor theory of hormone receptors and adenylyl cyclase interaction put forward by Cuatrecasas and coworkers. According to this theory the hormone receptors are not directly linked to the membrane adenylyl cyclase but following binding of hormone to the receptor the affinity of these two membrane molecules for each other is increased and they diffuse laterally to combine into a complex leading to the activation of the enzyme (Cuatrecasas, 1974; Bennett et al., 1975). This model would account for the observed lag period between binding of

a hormone and activation of adenylyl cyclase and explain how several different hormones that bind to different receptors can all stimulate the same enzyme. The binding of some hormones shows the phenomenon of negative co-operativity which implies decreased affinity of binding with higher concentration of hormone and receptor occupancy caused by an interaction between receptor molecules (Bradshaw & Frazier, 1977; Catt & Dufau, 1977). In the case of insulin ferritin-labelled insulin can indeed be seen on electron microscopy to bind both in a diffuse pattern and in clusters on the cell surface (Orci et al., 1975).

8.1.5. Cellular adhesion. Specific receptor molecules of the type described above recognize and bind other molecules of varying size such as small neurotransmitter molecules, polypeptide hormones and protein antigens. The study of cellular recognition of larger particles such as other cells involves an experimental approach somewhat different from that used for the study of molecular receptors. An example of this kind of recognition is provided by the phenomenon of "sorting out" of dispersed embryonic cells. These cells will reaggregate in a tissue-specific way and will even form an organ-specific pattern. The experimental techniques include the assaying of reaggregation of dispersed cells, often done in shaking cultures to encourage cell contact (Moscona, 1962) and measurement of the

binding of radioactively labelled dispersed cells to preformed cellular monolayers or aggregates (see Edwards, 1977). These methods have yielded information about patterns and specificities of binding and have also been used to work out kinetics and forces of adhesion but direct measurement of such forces by dispersion techniques have also been attempted (see Curtis, 1973). The experimental data can be analysed on the basis of physico-chemical considerations (see Curtis, 1973; Edwards, 1977). The differential adhesion hypothesis of "sorting out" was introduced by Steinberg (1963). According to this hypothesis cells which bind to their own kind with relatively low cohesive strength will envelope those that are bound together with stronger cohesive forces. The strength of adhesion between the different cell types will then be intermediate between the strengths of cohesion of the individual cell types. This hypothesis has not been proven but provides an explanation for the "onion" configuration frequently observed in the "sorting out" of dispersed embryonic cells.

8.1.6. Physicochemistry of cellular adhesion.

The biophysical factors that may be involved in cell adhesion have been reviewed by Curtis (1973). These include Brownian motion, electrostatic effects and long range forces such as London-van der Waals forces. Brownian motion energy seeks to randomize the distribution of particles and may lead to collisions

that can result in adhesion but will also tend to break up adhesions. In colloid chemistry the control of adhesion between particles by the interaction between the London dispersion forces of attraction and electrostatic forces of repulsion is described by the theory of Derjaguin and Landau, Verwey and Overbeek, the DLVO theory. This theory predicts that there are two spacings of adhesion, firstly strong adhesion at close molecular contact and secondly a weak reversible adhesion with a fluid-filled gap of 10-20 nm between the surfaces. This second type of adhesion is caused by the fact that the repulsive force decreases more rapidly with distance than the attractive force and thus the attractive force becomes greater than the repulsive force at two separations (Curtis, 1973; Edwards, 1977). Living cells carry a negative surface charge and there is some evidence that this may provide a repulsive force opposing adhesion although this could not always be confirmed (see Curtis, 1973). There is no direct proof that the interaction of forces described by the DLVO theory occurs in cellular adhesion but some measurements of adhesive forces would seem to fit the theoretical predictions (Curtis, 1973; Edwards, 1977). Several other physical factors such as hydrodynamic forces may influence cellular adhesion, these have not often been taken into consideration but they may cause delayed adhesion or separation with increased viscosity of the medium that has to leave or enter gaps between cells (Curtis, 1973).

8.1.7. Cell ligands. Both Curtis (1973) and Steinberg (1963) are of the opinion that physicochemical forces are sufficient to explain adhesion and segregation of cells and that there is no need for bridging mechanisms or molecular specificity. This view, that ascribes adhesion to the bulk properties of cell surfaces, is not shared by workers who maintain that adhesion is mediated by individual molecules. Moscona (1962) introduced the concept of "cell ligands" to explain the tissue-specific aggregation of dispersed embryonic cells. He proposed that the cells produce specific surface components that are released into the surrounding medium and can then mediate cell to cell attachment. This was based on the observation that supernatants of cells kept at a high rotating speed, when shearing forces are great enough to prevent aggregation, stimulated the aggregation of cells of their own kind but not other cells. Subsequently glycoproteins with the characteristics of membrane proteins that bind specifically to trypsinized homologous cells have been isolated from culture supernatants (Hausman & Moscona, 1975; Balsamo & Lilien, 1974). It was also shown that proflavine can inhibit in a fairly specific way the synthesis of an "aggregating factor" without affecting agglutinability by Concanavalin A or wheat germ haemagglutinin (Hausman & Moscona, 1973). Many of these experiments have been performed using cells that have been dispersed by trypsinization and as pointed out by Curtis (1973)

the requirement for active metabolism for the aggregation of such cells may reflect the need for repair of the damage caused by trypsin rather than synthesis of a bridging molecule. The stimulating effect of cell-derived factors on the aggregation of trypsinized cells might be interpreted in a similar way. The requirement for a specific surface structure that is trypsin-sensitive does, however, tend to suggest that a degree of molecular specificity is involved in the aggregation. A glycoprotein that causes selective aggregation of dissociated sponge cells has been identified by Margoliash et al. (1965). Curtis & van de Vyver (1971) found that sponges produced soluble factors that diminish the adhesion of cells of unlike kind whilst increasing the adhesiveness of homologous cells and this behaviour was considered to make it unlikely that these factors were bridging agents but it does not exclude the possibility that they are surface recognition molecules. Rutishauser et al. (1976) have recently studied a surface protein of chick embryonic retinal cells which is also released into the culture medium. This protein is composed of two subunits one of which has a great tendency to form a dimer. Antibodies against this component bind to the cells and prevent aggregation. On the basis of their observations the authors propose the hypothesis that in cell adhesion the non-binding component is cleaved off and binding achieved by means of dimerization of the binding component. In these experiments the cells were dispersed into

single cell suspensions by pipetting. It has usually been found that dispersed embryonic cells bind with preference to cells of their own type (Moscona, 1962) but binding to different cell types also occurs sometimes with a degree of specificity according to patterns of organogenesis (Barbera et al., 1973). Binding of embryonic cells is, however, not species specific (Moscona, 1962) and McClay & Gooding (1978) have recently shown that major histocompatibility antigens are unlikely to be involved in this type of recognition.

8.1.8. The role of carbohydrates in cellular adhesion. The importance of surface carbohydrates in cellular recognition has been emphasized in recent years. Moscona (1962) had found that treatment of dispersed embryonic cells with periodate inhibited their reaggregation and certain surface sugars seem to play a specific part in mammalian fertilization (Oikawa et al., 1973). It was suggested several years ago that surface glycoproteins determined recirculation patterns and "homing" of lymphocytes (Gesner & Ginsburg, 1964; Woodruff & Gesner, 1969; Woodruff, 1974). Some doubt has now been cast on this hypothesis and sialic acid residues on the lymphocyte surface do not appear to have the recognition function previously ascribed to them. Trypsin-sensitive glycoproteins might, however, still be of importance (Ford et al., 1976). The pattern of surface carbohydrates changes

in relation to contact inhibition of normal cells in culture (Nicolson & Lacorbiere, 1973). On the basis of studies indicating the importance of surface carbohydrates and their active synthesis in cellular adhesion Roseman (1970) developed the hypothesis that adhesion is mediated by the combination of glycosyltransferases on one cell with their substrate on another cell. He also suggested that after completion of the glycosyltransferase reaction the cells could detach again. Roth et al. (1971a and b) were able to show that treatment of neural retinal cells with glycosidic enzymes reduces their specific aggregation and there was glycosyltransferase activity on the cell surface. Transfer of labelled galactose by the galactosyltransferase of one cell onto an acceptor on an adjacent cell appears to occur after the cells have come into contact with each other (Roth & White, 1972). There is thus some circumstantial evidence supporting Roseman's hypothesis but it has so far remained inconclusive. The possibility of a different type of recognition mediated by cell surface carbohydrates has recently come to light with the discovery of lectin-like molecules in animal cell membranes. Such molecules have been isolated from slime molds (Reitherman et al., 1975), electric organs of the electric eel (Teichberg et al., 1975), chick embryo fibroblasts (Yamada et al., 1976), rabbit liver (Ashwell & Morell, 1974) and calf heart and lung

(DeWaard et al., 1976). Many of these molecules have been shown to agglutinate erythrocytes and have a specificity for galactosides. A role in cell adhesion is suggested by the observation that the appearance of two lectins on the surface of the slime mold Dictyostelium discoideum coincides with its differentiation from a vegetative (non-cohesive) state into a cohesive state. Simultaneously the cells become more agglutinable by the lectins and also by a plant lectin, Ricinus communis agglutinin, which is specific for D-galactose (Reitherman et al., 1975). The glycoprotein from chick embryo fibroblasts was found to restore normal adhesiveness and contact inhibition to malignantly transformed fibroblasts (Yamada et al., 1976). The lectins of leguminous plants have been shown to bind in a specific way to capsules (Dazzo & Hubbell, 1975) and cell wall polysaccharides (Planqué & Kijne, 1977) of Rhizobia and may thus mediate the attachment of these nitrogen-fixing bacterial symbionts to the plant roots.

8.1.9. The role of divalent cations in cellular adhesion. Cellular adhesion has frequently been found to require divalent cations, in particular Ca^{++} and Mg^{++} . These cations have many different effects in biological systems (Williams, 1970) and it is not known how they promote cellular adhesion. The suggested possibilities include a bridging action perhaps indirectly by binding of another bridging molecule or, alternatively, a reduction in electrostatic repulsion (see Curtis, 1973).

8.1.10. Relative importance of physical factors and molecular specificity. The relative importance of bulk properties and physical forces versus specific molecular binding in cellular adhesion is difficult to assess at present. It would seem likely that long-range physical forces are important in determining the balance between repulsion and attraction in the first stages of attachment but that binding itself relies on specific molecular recognition. It is hard to see how some complex cellular junctions which seem to depend on a specific surface pattern (Nicolson et al., 1977; see also Curtis, 1973) could not have molecular specificity.

8.2 Specific receptors on the macrophage surface.

8.2.1. The Fc-receptor. Immunoglobulin and complement function as recognition molecules, or opsonins, for phagocytic cells and mediate the binding of particles ranging in size from antigen-antibody complexes to whole cells coated with antibody and complement. Antibodies can be bound to the macrophage surface even when not combined with antigen and are then known as cytophilic antibodies. This was first demonstrated by Boyden & Sorkin (1960) who showed that rabbit spleen cells adsorbed antibody from serum and could then specifically bind radiolabelled antigen. Boyden (1964) subsequently did similar experiments using peritoneal exudate cells from guinea-pigs and found that only the adherent cells could bind antibodies. This was confirmed by Uhr (1965) who

found that both macrophages and lymphocytes could bind antigen-antibody complexes but only macrophages were capable of binding antibody alone. The macrophage receptor for antibody can be studied by using antigen-antibody complexes, usually antibody-coated particles such as erythrocytes, or Boyden's original technique when antigen is added as an indicator after exposure of the macrophages to the antibody. More recently purified radiolabelled immunoglobulins, often of myeloma origin, have been employed and this is required for kinetic studies. Using Boyden's method and electrophoretically separated immunoglobulins, Berken & Benacerraf (1966) demonstrated that in the guinea pig immunoglobulin G2 was cytophilic whilst IgG1 was not. It has now been shown, using the more sensitive assay of binding of radiolabelled (^{125}I) immunoglobulins, that both monomeric IgG1 and IgG2 bind to guinea pig macrophages but the number of receptors per cell and avidity of binding is lower for IgG1 than IgG2 (Leslie & Cohen, 1976). Inhibition studies showed that the receptors for each subclass are not identical but probably related as indicated by partial inhibition of binding of IgG1 by IgG2. Binding of IgG1 was enhanced by complex formation and complexes formed with IgG1 or IgG2 have been found to bind equally well (Shinomiya & Hoyama, 1976). Subclass specificity of immunoglobulin-receptors on macrophages has also been noted in other species. Thus human monocytes

bind human myeloma IgG1 and IgG3 strongly but binding of IgG2 and IgG4 is weak (Hay et al., 1972). In the mouse only IgG2a is bound non-aggregated, very little binding is observed with monomeric IgG2b, IgG1 or IgG3 but aggregates of IgG2b and IgG1 bind efficiently (Walker, 1976c; Heusser et al., 1977). It was concluded on the basis of inhibition experiments and trypsin-sensitivity that mouse macrophages have two different receptors for IgG, one specific for IgG2a (aggregated or non-aggregated) only, which is trypsin-sensitive, and another that binds monomeric IgG2b as well as aggregates of this and other subclasses (Heusser et al., 1977).

From a functional point of view it would be expected that the Fc-portion of IgG bound to the macrophage surface whilst the Fab-portion is combined with the antigen or free to do so. This has been found to be the case in experiments using enzyme-derived fragments of IgG in binding and inhibition studies (LoBuglio et al., 1967; Dissanayake & Hay, 1975). It appears to be the C_H3 domain that is mainly involved in binding (Yasmeen et al., 1973; Okafor et al., 1974) although some authors have found that the C_H2 domain also plays a minor (Ciccimara et al., 1975) or even major part (Alexander et al., 1976).

The number of binding sites for IgG (Fc-receptors) is increased in activated macrophages (Arend & Mannik, 1973) and

this, along with species differences, may account for the difference between the number reported for rabbit alveolar macrophages, about 2×10^6 per cell (Arend & Mannik, 1973; Phillips-Quagliata et al., 1970) and a mouse macrophage cell line, 10^5 sites per cell (Unkeless & Eisen, 1975). Binding to the Fc-receptor is enhanced by immune complex formation with divalent or polyvalent haptens and this enhancement was found to be optimal at equivalence, when lattice formation is favoured (Phillips-Quagliata et al., 1970). These findings have been confirmed more recently. Thus Segal & Hurwitz (1977) showed that oligomers of IgG, cross-linked with a bivalent affinity label, bound with increasing affinity to the Fc-receptor according to size of the oligomer and the kinetics suggested multi-stage binding. The greater avidity of binding of IgG aggregates seems to be caused by a slower dissociation rate with increasing aggregate size whilst the association rate stays the same as for monomeric IgG (Knutson et al., 1977). Binding of IgG does not appear to require the presence of Ca^{++} (Berken & Benacerraf, 1966; LoBuglio et al., 1967).

8.2.2. The nature of the Fc-receptor. Early studies had indicated that the Fc-receptor was resistant to the action of trypsin but was destroyed by phospholipases (Howard & Benacerraf, 1966; Davey & Asherson, 1967; Arend & Mannik, 1973). More recent work has demonstrated that the

receptor for IgG2a on mouse macrophages is sensitive to trypsin whilst the other Fc-receptor, binding aggregates of several IgG-subclasses as well as heterologous IgG is not affected by this enzyme (Unkeless, 1977; Heusser et al., 1977). Attempts have been made to test whether the Fc-receptor is linked to known surface antigens of the macrophage. In the mouse Fc-receptors do not seem to be closely linked to the major histocompatibility antigens as indicated by studies using anti-H-2 antibodies under conditions where either blocking or "antigenic modulation" is achieved (Kerbel, 1976; Schlesinger & Chaouat, 1975). Fab₂-fragments of anti-macrophage antibodies have also been found not to affect binding to the Fc-receptor (Holland et al., 1972).

8.2.3. Functions of the Fc-receptor. The most commonly observed function of binding to the Fc-receptor is phagocytosis but cytotoxicity without phagocytosis also occurs and IgG-mediated binding of antigen may be important in the secondary immune response. Recent studies have indicated that different Fc-receptors may not mediate the same functions. Thus Walker (1977) found that IgG2a, which can bind both non-aggregated and aggregated, mediated only phagocytosis but not extracellular lysis of chicken erythrocytes by a mouse macrophage cell line. Binding with IgG2b, bound only when aggregated, led to both phagocytosis and extracellular cytolysis.

Portis & Coe (1978) have recently studied hamster macrophages and demonstrated that IgG1 was bound with greater avidity than IgG2 but ingestion of IgG1-coated particles was less efficient thus perhaps suggesting a role for this antibody subclass in antigen presentation. The importance of binding of antigen to cytophilic antibody in the secondary immune response particularly at low antigen concentrations was demonstrated in in vitro studies by Cohen et al. (1973). It is interesting to note in this context that a high proportion of macrophages can be shown to carry surface immunoglobulin when freshly isolated from the animal, this is rapidly lost and new immunoglobulin from the surrounding medium taken up showing that IgG bound to macrophages is freely exchangeable (Loor & Roelants, 1974). In vivo the Fc-receptor may also be involved in the removal of senescent erythrocytes from the circulation as suggested by the studies of Kay (1975). An additional and, phylogenetically, possibly older function of the Fc-receptor is indicated by the finding that C-reactive protein binds to this receptor (Mortensen & Duszkievicz, 1977).

8.2.4. Receptors for immunoglobulins other than IgG.

The binding to macrophages of immunoglobulins of other classes has been far less studied than the binding of IgG. Lay & Nussenzweig (1969b) demonstrated binding of sheep erythrocytes coated with homologous 19S antibodies (IgM) to mouse macrophages

whilst no binding occurred if the erythrocytes were sensitized with heterologous 19S antibodies. The avidity of binding was lower than with IgG-coated particles and there was no phagocytosis. The receptor appeared to be separate from the receptor for IgG, it was resistant to trypsin but Ca^{++} was required for attachment. Myeloma-proteins of the immunoglobulin classes A and M are not cytophilic for a mouse macrophage cell line (Walker, 1976c) but myeloma IgM mediates phagocytosis of chicken erythrocytes by these macrophages without leading to extracellular cytolysis (Walker, 1977). IgM may thus help to focus antigen on the macrophage surface in the primary immune response and play some role in the scavenging of antigenic particles during that period. It has recently been shown that complexes or aggregates of IgE are bound to rat macrophages and these then show increased activity of lysosomal enzymes and become cytotoxic against schistosomes suggesting a role for IgE and macrophages in the defence against these helminths (Capron et al., 1977).

8.2.5. The complement receptor. A receptor for complement was first described on human monocytes by Huber et al. (1968) and on mouse peritoneal macrophages by Lay & Nussenzweig (1969a). These studies showed that this receptor could not be blocked by IgG and was therefore separate from the Fc-receptor, it was destroyed by trypsin and binding required Mg^{++} but not Ca^{++} .

When particles are opsonized with complement the attachment to macrophages is probably mediated by the C3d fraction of the third component of complement (Rabellino & Metcalf, 1975) but it has been shown that human monocytes can bind particles coated with either C3b, C3d or C4 (Ross & Polley, 1975). Binding to the complement receptor does not always lead to phagocytosis. When normal mouse macrophages are used complement only mediates attachment but IgG and unblocked Fc-receptors are required for subsequent phagocytosis (Mantovani et al., 1972). Following stimulation with thioglycollate the function of the receptor is altered so that attachment leads to ingestion (Bianco et al., 1975). This might be linked to an increase in the number of receptors. Redistribution of the receptors in the macrophage membrane may be important for their function as indicated by the finding of Atkinson et al. (1977) that cytochalasins inhibit rosetting with complement-coated erythrocytes. The C3-receptor can also mediate phagocytosis by activated alveolar macrophages of particles opsonized in the absence of immunoglobulin via the alternative pathway (Stossel, 1973). An additional function of C3b-receptors is suggested by the recent observation that C3b (in large concentrations) stimulates the secretion of lysosomal enzymes (Schorlemmer & Allison, 1976) and renders macrophages cytotoxic (Schorlemmer et al., 1977a).

The studies of Schlesinger & Chauat (1975) indicate that the receptor for complement-coated red blood cells is closely related to the H-2 antigens in the mouse, particularly the Ia-antigens.

8.2.6. Binding of other opsonins. Serum contains other and often not very well defined opsonic factors in addition to immunoglobulins and complement and receptors on macrophages for some of these have been investigated. Nelson (1970) has described a fast-moving α_1 -globulin that is present in increased concentration in mouse serum in the early phase of a primary immune response. This protein reacts with some specificity with the antigen and binds to a trypsin-sensitive receptor on the macrophage surface. An α_2 -macroglobulin with apparent opsonic activity in vitro as well as in vivo has been identified in rat and human serum (Piasano & Di Luzio, 1970; Allen et al., 1973; Di Luzio et al., 1974). This protein, which has been called humoral recognition factor (HRF), is present in decreased concentration in certain disease states, including malignancies; it is chemotactic for macrophages and has an antitumour effect if given intralesionally presumably by attracting and perhaps by activating macrophages (Di Luzio et al., 1974; Mansell et al., 1976).

8.2.7. Binding of lymphokines. Several lymphokines affect the function and behaviour of macrophages, these include

migration inhibition factor (MIF), macrophage activating factor (MAF) and chemotactic factor (CF). A trypsin-sensitive receptor specific for MIF was first described on peritoneal exudate macrophages from guinea pigs by Leu et al. (1972). This receptor appeared at that time to be absent from alveolar macrophages but other workers have since detected an MIF-receptor on alveolar macrophages from rabbits and guinea pigs (see Moore & Myrvik, 1977). Binding of MIF is dependent on a fucose residue on the macrophage surface as shown by the inhibition of binding by α -L-fucose and destruction of the receptor by α -fucosidase (Remold, 1973; Rocklin, 1976). Stimulation by lymphokines of production of the second component of complement is also inhibited by α -L-fucose (Littman & Ruddy, 1977) and this sugar as well as α -L-rhamnose and L-xylose inhibited the chemotactic response of macrophages to lymphokines which was not affected by several other sugars (Amsden et al., 1978). Surface glycoproteins and in particular their sugar residues appear thus to function as receptors for lymphokines on macrophages. Macrophage activating factor may be identical with MIF (David & Remold, 1976) but the chemotactic factor appears to be a different molecule (Ward et al., 1970).

8.2.8. Binding of other proteins. Specific receptors have been described for three other proteins on the macrophage. MacSween & MacDonald (1969) studied the binding of transferrin

to macrophages which has implications for the metabolism of iron as mentioned earlier (see page 46). Van Snick & Masson (1976) have shown that human lactoferrin binds in a specific way to mouse macrophages but the functional importance of this finding is not clear. A specific receptor for fibrin has recently been described by Sherman & Lee (1977) and Ca^{++} -dependent binding of fibrinogen/fibrin to the macrophage surface was also observed by Colvin & Dvorak (1975). There are indications that these coagulation factors may play a role along with complement factors in adherence and spreading of macrophages in vivo as well as in vitro (Colvin & Dvorak, 1975; Bianco et al., 1976) and they could also be involved in the inhibition of macrophage migration (Meade et al., 1976).

8.2.9. Hormone receptors. Macrophages have also been shown to possess receptors for various hormones but their biological function is not well known at present. The binding of insulin to receptors on human monocytes has been studied in some detail (Beck-Nielsen et al., 1977). Although insulin is not required for the transport of glucose across the macrophage membrane (Gee et al., 1974) it may cause a decrease in the density of Fc-receptors (Rhodes, 1975b). There is also evidence for a β -adrenergic receptor on guinea pig macrophages and they respond briefly to isoprotorenol or aminophylline by an increase in cyclic AMP but become refractory to further

stimulation within 3 minutes (Higgins & David, 1976). Glucocorticosteroids have been reported to inhibit some aspects of macrophage function, including binding to Fc- and complement receptors (Schreiber et al., 1975) and "arming" by lymphocyte products (Dimitriu, 1976) but have only very slight effects on the ability of macrophages to phagocytose and kill bacteria (van Zwet et al., 1975).

8.3 Non-specific recognition by macrophages.

The term non-specific recognition is used here to describe recognition phenomena that have not so far been shown to be mediated by specific receptor molecules but does not imply that they might not have a molecular basis. There are numerous examples of this type of recognition by mononuclear phagocytes. The firm adherence to different surfaces and phagocytosis of a variety of inert particles such as polystyrene microspheres and starch granules is one of the defining characteristics of mononuclear phagocytes (van Furth et al., 1972; Stossel, 1975). It is well known that macrophages take up different kinds of antigen for subsequent "presentation" to lymphocytes (Nossal et al., 1964; Unanue & Askonas, 1968; Unanue, 1972) and surface binding of protein antigens to macrophages independent of antibody has been demonstrated (Unanue & Calderon, 1975; Gallily & Bar-Eli, 1976; Loor & Roelants, 1974).

8.3.1. Binding of foreign or aged cells. Since

the days of Metchnikoff there have been many reports on the binding of foreign or aged red blood cells to mononuclear phagocytes and erythrophagocytosis. Thus Perkins & Leonard (1963) found that induced mouse peritoneal macrophages discriminated between homologous and heterologous erythrocytes and the degree of phagocytosis was greatest with red blood cells from species least related to the mouse. Normal mouse macrophages phagocytose aged but not fresh human red blood cells (Stuart & Cumming, 1967). Maruta & Mizuno (1971) also came to the conclusion that erythrocytes from different species become more susceptible to phagocytosis by mouse macrophages with increased foreignness and age. These experiments were all done in the presence of serum which could have contributed opsonic factors. There is, however, evidence that damaged red blood cells can bind to macrophages in the absence of serum. Old guinea pig erythrocytes bind to homologous macrophages without requirement for serum, no binding occurs with fresh erythrocytes (Vaughan & Boyden, 1964). Rabinovitch (1967_a) showed that erythrocytes from various species that had been modified by heat or chemicals bound to mouse macrophages in an assay performed in buffered saline; untreated erythrocytes were not bound. There is also recent evidence showing that normal macrophages recognize and destroy xenogeneic but not allogeneic or syngeneic

fibroblasts (Cabilly & Gallily, 1977).

8.3.2. Binding of micro-organisms. A wide range of micro-organisms is taken up by macrophages. Again in the vast majority of experiments serum has been used and the importance of opsonins is often stressed (Jenkin & Rowley, 1961; Auzins & Rowley, 1963; van Furth & van Zwet, 1975). In several instances phagocytosis of micro-organisms by mononuclear phagocytes has been observed in vitro in the absence of specific antibodies but the medium has nearly always contained varying amounts of homologous or heterologous serum though often heat-inactivated to exclude complement activity. The micro-organisms that are phagocytosed by rodent macrophages under these conditions include: Salmonella typhimurium (Furness, 1958; Gelzer & Suter, 1959), Brucella (Holland & Pickett, 1958), Listeria monocytogenes (Fowles et al., 1973), Pseudomonas pseudomallei (Kishimoto & Eveland, 1976), Nocardia asteroides (Beaman & Smatters, 1976) and Coxiella burnetii (Kishimoto & Walker, 1976). Heat-inactivated normal serum (which could still contain opsonins other than specific immunoglobulins or complement) was used in all of these studies apart from those dealing with Pseudomonas pseudomallei and Coxiella burnetii where the serum was not heat-inactivated. Pneumocystis carinii becomes attached to rat or mouse macrophages in the presence of heat-inactivated fetal calf serum but phagocytosis only occurs if specific antiserum is added

(Masur & Jones, 1978). It is an old observation that capsulated bacteria are less readily phagocytosed than non-capsulated ones (see Dubos, 1945; Wood, 1960). The capsulated organisms appear to resist the attachment phase of phagocytosis (see Jones, 1975). Smooth strains of Salmonella are also more resistant to phagocytosis than rough strains where part of lipopolysaccharide is missing (Friedberg & Shilo, 1970). Recognition of bacterial products is also reflected in a chemotactic response to certain lipids (Tainer et al., 1975; Russell et al., 1976) and peptides (Schiffman et al., 1975a) of bacterial origin. Many viruses are taken up by macrophages (Allison, 1974) which is not surprising since viruses live and multiply intracellularly. There is, however, recent evidence that calf macrophages can recognize parainfluenza-3 antigen on the surface of infected cells and have a cytotoxic effect against such cells (Probert et al., 1977).

8.3.3. Binding of tumour cells and other rapidly dividing cells. A vast literature has accumulated in the last few years dealing with the cytotoxic effects of macrophages on tumour cells. In in vitro cytotoxicity tests the macrophages can be seen to accumulate around their targets and close cell contact is generally found to be required for cell killing (Hibbs et al., 1972; Keller, 1974; Evans & Alexander, 1976). The macrophages have to be "activated" in order to achieve this

effect. Malignant cells of different species appear equally susceptible to the cytotoxic effect of activated rat or mouse macrophages (Keller, 1976) but certain surface characteristics related to the rate of proliferation and degree of contact inhibition are important. Thus it has been found that transformed cells that still show contact inhibition are not killed by macrophages (Hibbs, 1973) and Keller (1976) has shown that activated macrophages inhibit the growth of most rapidly proliferating cells regardless of whether they are derived from normal or malignant tissue but malignant cells are usually more easily killed although syngeneic embryonic cells can also be susceptible (Jones et al., 1975). In most of these experiments the recognition phase involved in the cytotoxicity has not been studied separately and they are all performed in the presence of serum but Piessens (1978) has recently found that the binding of hepatoma cells to monolayers of guinea pig macrophages did not require serum.

8.3.4. Binding of lymphocytes. The binding of lymphocytes to macrophages provides an exception to the general rule that macrophages recognize non-self or altered self but are indifferent towards normal self. Lipsky & Rosenthal (1973 and 1975a) showed that lymphocytes (both B and T) from guinea pigs bind in vitro to macrophages in the absence of antigen. This binding was species specific but did occur between lymphocytes and macrophages from different strains of guinea

pigs. This type of binding was not very stable or long-lived. In the presence of antigen the attachment of immune lymphocytes to macrophages is greatly enhanced and they remain bound for up to 72 hours in culture at which time they have started to synthesize DNA (Lipsky & Rosenthal, 1975b). This antigen-dependent binding, which thus seems to be of great importance for the stimulation of lymphocytes, is strictly dependent on histocompatibility between the lymphocytes and macrophages (Rosenthal et al., 1976). Braendstrup & Werdelin (1977) have recently described the formation of clusters of lymphocytes around antigen-pulsed macrophages. Only immune lymphocytes form a direct contact with the macrophages but non-immune lymphocytes join the cluster by binding to the immune lymphocyte. The direct binding to macrophages appears to be limited mainly to immune T-lymphocytes (Petri et al., 1978).

8.3.5. The role of physical factors in non-specific phagocytic recognition. Little is known about the mechanisms behind non-specific recognition by macrophages and when micro-organisms or cells are involved it is generally not known whether a specific molecular entity is being recognized, the exception being the antigen-dependent binding of lymphocytes which appears to be based on mutual recognition of major histocompatibility antigens. Mudd et al. (1934) explained the attachment of particles to phagocytic cells on the basis of

relative interfacial tension between the particles and the suspending medium on one hand and particles and phagocytes on the other. The enhancing action of immune serum was attributed to its ability to change the surface properties of the particles. This view has become less popular with the discovery of specific receptors on phagocytes as well as other cells; there is, however, some recent work emphasising the importance of these physico-chemical factors. Thus van Oss & Gillman (1972a and b) measured the contact angle that a drop of saline made with a monolayer of polymorphonuclear leucocytes or lawn cultures of bacteria as an indication of hydrophobicity and found that bacteria that were more hydrophobic than the cells were phagocytosed but hydrophilic bacterial capsules were associated with very low levels of phagocytosis. Specific antiserum increased the hydrophobicity of encapsulated bacteria and rendered them more susceptible to phagocytosis and if complement was also added both hydrophobicity and phagocytosis were even further enhanced (Van Oss & Gillman, 1972b). Recently Tagesson et al. (1977) have used liposomes composed of phosphatidylcholine, cholesterol and diacetylphosphate as models for plasma membranes. These liposomes behave similarly to plasma membranes when exposed to steroids, lytic proteins, polyene antibiotics and complement. Addition of aggregated IgG or Salmonella typhimurium opsonized with IgG caused the release of an incorporated probe whilst little or no release was

observed with non-aggregated IgG, S. typhimurium coated with $F(ab')_2$ -fragments or unopsonized Salmonellae. Even though these correlations exist there is no direct proof that the physicochemical factors studied by Van Oss & Gillman and Tagesson et al. are indeed involved in the attachment of particles to phagocytes or whether these factors could alone be responsible. Wilkinson (1976a) has proposed that many chemotactic agents exert their effect by virtue of their hydrophobicity in a manner similar to the action of anaesthetic drugs. This is based on experiments showing that many proteins that have been made hydrophobic by denaturation are chemotactic whereas the native proteins often are not. Bacterial lipids were also found to be chemotactic for macrophages (Russell et al., 1976). In addition Wilkinson (1975 and 1976a) has shown that the chemotactic response of human neutrophils and monocytes towards casein, endotoxin activated serum or denatured albumin is depressed if the cells have been treated with bacterial phospholipases or cholesterol-binding toxins whilst treatment with trypsin, pronase, α -fucosidase or neuraminidase had no effect.

8.3.6. The role of specific binding sites. The greater degree of uptake by macrophages of alumprecipitated or heat-aggregated bovine serum albumin (BSA) as compared with

soluble BSA (Nakano, 1976; Mehl & Lagunoff, 1975) might be explained in a similar way to that just described. Mehl & Lagunoff (1975), however, interpret their results in terms of a specific binding site that soluble and aggregated BSA compete for. The calculated number of binding sites is lower for aggregated than soluble BSA but the affinity of binding is greater. Recent studies seem to indicate that a certain type of synthetic chemotactic agents (formylmethionyl oligopeptides) binds to specific receptor sites on polymorphonuclear leucocytes and monocytes. The binding is reversible and saturable and inhibition studies with different formylmethionyl peptides indicate chemical specificity (Williams et al., 1977). Since formylation of methionine seems to be characteristic of protein synthesis in prokaryotic cells it has been suggested that this could represent a general recognition mechanism whereby phagocytes recognize bacteria (Schiffmann et al., 1975a; Williams et al., 1977).

Preferential binding to certain areas of the phagocyte surface was previously interpreted as a physical phenomenon of trapping as observed in so-called surface phagocytosis where bacteria become attached only to the periphery of surface-adherent phagocytes (Foley et al., 1959). This would in more recent years be seen as a reflection of uneven localization of receptors on the cell surface (Kaplan et al., 1975).

8.3.7. Nature of surface components involved in binding. In contrast to the findings of Wilkinson (1976a) described above other workers have found that binding or ingestion of particles by phagocytic cells is affected by treatment with trypsin or neuraminidase which would suggest that surface glycoproteins play a part in these phenomena. Rabinovitch (1967a) showed that attachment of modified erythrocytes to macrophages was reduced following trypsinization of the macrophages and exposure to trypsin also caused a decrease in phagocytosis of foreign erythrocytes by neutrophils (Bona et al., 1968). The reported effects of treatment with neuraminidase vary. Weiss et al. (1966) found that this caused an increase in adherence and ingestion of negatively charged particles by monocytes and this was attributed to a decrease in surface charge following removal of sialic acids and an increase in cellular deformability. Phagocytosis by neutrophils of starch particles (which are uncharged) was unaffected by pretreatment of the cells with purified neuraminidase (Noseworthy et al., 1972).

8.3.8. The role of surface charge in binding to phagocytes. The question of involvement of surface charge was approached in a different way by Nagura et al. (1977). These workers treated rat macrophages and the test particles (glutaraldehyde-treated sheep red blood cells) with either low ionic strength medium or protamine sulphate in order to increase

or decrease, respectively, the negative surface charge. They found that a decrease in negative charge was associated with increased phagocytosis, whilst increased negative charge had the opposite effect. The number of attached or ingested red blood cells was seen to fall in a linear fashion with an increase in the product of negative charge densities on the surfaces of the macrophages and the erythrocytes.

8.3.9. The role of divalent cations in phagocytic binding. Like many other cellular adhesion phenomena attachment to phagocytic cells has been found to require divalent cations. Thus both Ca^{++} and Mg^{++} but not Ba^{++} promoted the phagocytosis of latex and starch particles by sheep neutrophils with or without serum (Wilkins & Bangham, 1964). The authors concluded that these results could not be explained in terms of the DLVO - theory of colloid stability since the optimal concentrations for phagocytosis were too low to affect the surface charge of either cells or particles as was shown by electrophoresis. The inability of Ba^{++} to enhance phagocytosis in the same way as Mg^{++} or Ca^{++} makes it unlikely that a passive surface tension phenomenon is involved. In experiments testing the adherence of neutrophils to capillary tubes a requirement for Mg^{++} but not Ca^{++} was demonstrated (Bryant, 1969). Rabinovitch & De Stefano (1973) have studied the spreading of macrophages on

a surface in vitro, which probably has an adherence component. They found that Mn^{++} was the most effective of several metal ions promoting macrophage spreading followed by ions of Co, Ni, Zn, Cd, Mg and Fe in that order of efficiency. Ca^{++} had no effect on spreading. The effective concentrations were too low to have a significant effect on surface charge and this was also an unlikely mechanism in view of the specificity for ions which rather would imply an enzyme activation function. Divalent cations (Ca^{++} or Mg^{++}) have also been found to promote the antigen independent binding of lymphocytes to macrophages (Lipsky & Rosenthal, 1973).

8.3.10. The role of carbohydrates in non-specific phagocytic recognition. There are some indications that surface glycoproteins and recognition of carbohydrates may be involved in the attachment of micro-organisms to the macrophage surface. A glycoprotein isolated from chronic granulomas inhibits the phagocytosis of Staphylococcus albus (Bole et al., 1975) and this molecule seems to be able to bind to either the macrophages or the bacteria (Bole & Wright, 1976). Immunofluorescence showed that this material was present on and/or in macrophages in the granulomas (Bole & Wright, 1976). Cryptococcal polysaccharide can inhibit the attachment and ingestion of non-capsulated strains of Cryptococci to macrophages and can cause

detachment of organisms bound at 4°C (Kozel & Mastroianni, 1976).

8.3.11. Proposed chemical nature of binding.

Piessens (1977) has recently described that exposure to periodate increases the cytotoxicity of macrophages against hepatoma cells that have been pretreated with neuraminidase and galactosidase and suggested that in this case the attachment might be effected by the formation of Schiff bases. Rabinovitch (1970) has put forward the idea that in certain cases covalent bonding might occur between modified erythrocytes and macrophages.

9. MOTILITY

The rapidly occurring responses of phagocytes to surface stimulation are manifested as changes in cellular motility, viz. enhanced or inhibited migration and phagocytosis. The mediation of these responses is probably separate from the processes involved in the more slowly developing changes occurring in stimulation and activation of macrophages which are a subject of this study. A short description of mechanisms involved in motility responses is included here for comparison.

9.1 Chemotaxis and migration inhibition.

9.1.1. Chemotaxis. Macrophages in suspension or on a surface undergo random locomotion which, stimulated by the action of certain substances on the macrophage surface, is called chemokinesis. If, however, a concentration gradient of a stimulating substance is present directed movement occurs towards the increasing concentration and this is known as chemotaxis (Wilkinson, 1976b). Chemotaxis of leucocytes towards an inflammatory focus was observed in vivo before the turn of the century. The chemotactic response can be quantified in vitro by the double chamber method of Boyden (1962) where the migration of phagocytes through a millipore filter separating two chambers containing different concentrations of a chemo-attractant is assayed. A slide and coverslip technique where

the orientation of the cells can be observed directly allowing better distinction between chemokinetic and chemotactic effects has also been developed (Zigmond & Hirsch, 1973).

9.1.2. Chemotactic agents. Several substances have been shown to be chemotactic for macrophages. Some of these belong to the immune system, such as C5a and a chemotactic factor produced by lymphocytes (Snyderman & Mergenhagen, 1976). Macrophages coated with cytophilic IgG are attracted specifically by the appropriate antigen (Wilkinson, 1976b). Macrophages also migrate towards dead or damaged homologous cells (Bessis, 1974), casein and other proteins, particularly if these are denatured or substituted with non-polar groups (Wilkinson, 1976b), and synthetic N-formylmethionyl peptides that may be similar to chemotactic factors derived from bacteria (Schiffmann et al., 1975a and b). Lipids of bacterial origin have also been shown to be chemotactic for macrophages but not for polymorphonuclear leucocytes (Tainer et al., 1975; Russell et al., 1976).

9.1.3. Mechanisms of chemotaxis. As mentioned in the preceding chapter there is evidence that some chemotactic agents act on specific surface receptors but the hydrophobic nature of many chemoattractants has lead to the suggestion that they interact with the lipid bilayer of the plasma membrane in a non-specific way. Whatever the mechanism of interaction with

the plasma membrane may be, a leucocyte seems to be able to recognize a concentration gradient along its surface which results in orientation and directed movement (Zigmond, 1974). It has recently been suggested that a peptidase on the surface of macrophages and polymorphonuclear leucocytes may be important in the detection of the polarity of the gradient by cleaving peptide chemoattractants and thus freeing receptors for further reaction (Aswanikumar et al., 1976).

Exposure of phagocytic cells to some chemoattractants has recently been shown to lead to changes in ion fluxes across the plasma membrane and the intracellular distribution of Ca^{++} . Thus (Naccache et al., 1977) have found that N-formylmethionyl-peptides cause a rapid increase in the permeability of the plasma membrane of rabbit polymorphonuclear leucocytes to Na^+ followed by a smaller enhancement of K^+ -influx and Na^+ -efflux. There is also an increase in the membrane permeability to Ca^{++} and the intracellular exchangeable Ca^{++} -pool. Previous experiments had shown that both spontaneous and chemotactic movement is stimulated when the extracellular concentration of K^+ is raised moderately or an artificial increase in the intracellular concentration of K^+ is caused by an ionophore. A decrease in the extracellular concentration of Na^+ enhanced spontaneous movement but the response to the chemotactic agent was diminished (Showell & Becker, 1976). Several chemotactic agents were shown

by Gallin & Rosenthal (1974) to lead to a small but rapidly developing and sustained increase in the efflux of Ca^{++} from human granulocytes and the influx of Ca^{++} was decreased. At the same time the intracellular distribution of Ca^{++} was changed so that less Ca^{++} was present in the cytoplasm but the amount found in granules was increased. Even though there seems thus to be a reduction in the net influx of Ca^{++} the intracellular distribution and availability of Ca^{++} appears to be the important factor. This is suggested by the results of Wilkinson (1975) who found that removing divalent cations from the medium caused a decrease in the chemotactic response of human polymorphs and monocytes and the addition of divalent cation ionophores restored the responsiveness under such conditions. Gallin & Rosenthal (1974) had suggested that Ca^{++} acted on the contractile elements of the cell but according to recent results Ca^{++} may also influence the ion-permeability of the plasma membrane. It has been found that chemotactic agents cause electric hyperpolarization of the macrophage plasma membrane and that Ca^{++} is required for this effect (Gallin & Gallin, 1977). Cytochalasin B and colchicine have both been shown to inhibit chemotactic movement at certain concentrations (Zigmond & Hirsch, 1972; Becker et al., 1972; Wilkinson, 1976b) but these two agents had no effect on the membrane hyperpolarization which is a very early event occurring before visible changes in cell morphology (Gallin & Gallin, 1977). As colchicine affects only directed movement but not random motility, micro-

tubules appear to be concerned mainly with providing a vector of motion perhaps by assymmetric assembly (Gallin & Rosenthal, 1974; Wilkinson, 1976b).

From the data just described it appears that the chemotactic response could rely on mechanisms akin to those involved in stimulation of muscle contraction by neurotransmitters with rapidly occurring changes in ion fluxes and membrane potential leading to redistribution of intracellular Ca^{++} with activation of contractile elements.

9.1.4. Migration inhibition. The phenomenon of macrophage migration inhibition when macrophages are restricted in their locomotion can be seen as related to chemotaxis since the cells can be prevented from further movement when they have reached the focus of chemoattraction by a related stimulus. Thus lymphocytes produce both a chemotactic factor and a migration inhibition factor which have been shown to be separate molecules (Ward et al., 1970). Aggregated IgG or antigen-antibody complexes activate the complement cascade and thereby exert a chemotactic effect (Boyden, 1962; Snyderman & Mergenhagen, 1976) but antigen-antibody complexes can also cause migration inhibition (Spitler et al., 1969; Kotkes & Pick, 1975). Fibrin degradation products have been found to be chemotactic for neutrophils (Stecher, 1975) and there is some evidence that fibrin is sometimes involved in causing migration inhibition of macrophages (Meade et al., 1976). The recent

experiments of Snodgrass et al. (1977) give a neat illustration of the relationship between chemotaxis and migration inhibition. They observed enhanced motility of macrophages directed towards tumour cells. When the macrophages had reached their targets the movement slowed down again and the tumour cells were subsequently lysed. Migration inhibition is classically studied by the capillary tube method of George & Vaughan (1962) where the area of migration out of a capillary tube in the presence of the substance or factor to be tested is measured. More recent methods include measurement of migration into a semi-solid medium (Clausen, 1972) or out of a micro-droplet composed of such medium (Harrington, 1977).

9.1.5. Migration inhibition factor. Migration

inhibition has been most extensively studied using the lymphocyte-derived migration inhibition factor (MIF). This is a glycoprotein with a molecular weight of 3.5×10^4 to 5.0×10^4 (Remold & David, 1971). Some workers have found evidence for an antigen-specific migration inhibition factor that is active only in the presence of the appropriate antigen (Amos & Lachmann, 1970) but generally this is not the case (Remold et al., 1972). The presence of L-fucose on the macrophage surface appears to be essential for the action of MIF as L-fucose inhibited response to MIF and treatment of the macrophages with L-fucosidase had the same effect whilst exposure of MIF to this enzyme did not

destroy its activity (Remold, 1973). The action of MIF has been found to be enhanced by various esterase inhibitors and chemicals that affect cell surface proteins and this has led to the suggestion that an esterase on the macrophage surface modulates the response to MIF thus serving as a control mechanism (David & Remold, 1976; Remold, 1977).

9.1.6. Mechanisms of migration inhibition. Remold-O'Donnell & Remold (1974) showed that a fraction of a lymphocyte supernatant rich in MIF caused enhanced activity of adenylyl cyclase in macrophages after 24 hours of incubation. This has, however, subsequently been shown not to be followed by an increase in the concentration of cyclic AMP (Higgins et al., 1976; Pick, 1977) and it has therefore been concluded that cyclic AMP does not play the role of a second messenger in the action of MIF. The MIF-rich fraction used by Remold and colleagues has also been found to stimulate macrophage metabolism and function and may thus be identical with the lymphocyte-produced factor called macrophage activating factor to describe its action (David & Remold, 1976). This will be dealt with in a later chapter.

MIF increases the adherence of macrophages to a glass or plastic surface (Nathan et al., 1971; David & Remold, 1976) and Curtis (1973) has suggested that this might be the basis of the migration inhibition. Dvorak et al. (1972) have

studied the possibility that MIF could induce changes in the macrophage surface, particularly a carbohydrate "cell coat". Their electron microscopy studies showed that macrophages exposed to MIF establish cell to cell contacts through long villous processes. A dense surface material seen on untreated cells as patches and thin layers particularly on blunt pseudopods was absent at points of cell contact but remained intact elsewhere. MIF enhances membrane ruffling and phagocytosis (David & Remold, 1976) and does thus not seem to have an overall inhibitory effect on macrophage motility. In some cases inhibition of migration could be mechanical such as when the cells apparently become trapped in a mesh of fibrin (Meade et al., 1976). In this context it is interesting to note that exposure of macrophages coated with cytophilic antibody to antigen does not result in migration inhibition whilst antigen-antibody complexes do inhibit macrophage migration and this is dependent on the Fc-portion of IgG (Kotkes & Pick, 1975).

9.2 Phagocytosis.

Following attachment of a particle to the surface of a phagocyte engulfment will take place, given the appropriate conditions. Ingestion is an energy-requiring process. Phagocytosis by macrophages can be arrested at the attachment phase by keeping the cells at a low temperature (Rabinovitch, 1967b) and inhibitors of energy metabolism cause a marked depression in phagocytosis (Karnovsky et al., 1970). The

process of phagocytosis as observed by transmission electron microscopy appears to involve the extension of pseudopods around the particle until they meet and fuse thereby completely surrounding the particle (see Stossel, 1976). The three-dimensional pictures obtained by scanning electron microscopy suggest that the particle "sinks in" and fine finger-like projections are seen linking the particle to the macrophage surface at the rim of the hollow (Kaplan et al., 1975). At the same time the surrounding surface of the macrophage shows increased formation of pseudopods and veil-like flaps (Walters et al., 1976).

Recent studies and theories on the process of phagocytosis have taken into account current knowledge and hypotheses about the plasma membrane and the role of cytoskeletal elements in cellular motility and regulation of cell surface architecture. Thus some workers have emphasized the role of surface receptor molecules and their movement within the membrane while others have investigated the contractile proteins of phagocytes and their response to exposure of the cells to particles.

9.2.1. The role of surface receptors in phagocytosis.

Griffin et al. (1975) developed the so-called "zippering" hypothesis of phagocytosis. In their experiments they allowed particles opsonized with either complement or IgG to attach to thioglycollate-induced macrophages in the cold. On warming

to 37°C the macrophages would normally ingest the attached particles. No phagocytosis takes place when the macrophages are warmed up if either the opsonizing complement outside the initial zone of attachment is removed by mild trypsinization or the Fc-receptors that are still free are blocked by anti-macrophage IgG. This implies that contact at one point does not trigger off an "all or none" response of engulfment but that a sequential, circumferential attachment of the macrophage plasma membrane receptors to the particle - "zippering" - occurs. These conclusions were confirmed by the later experiments using lymphocytes with capped anti-immunoglobulin IgG (Griffin et al., 1976). The authors propose that the binding to a receptor causes an alteration in the shape of the receptor molecule that leads to localized aggregation of adjacent microfilaments thus pushing forward pseudopods and allowing approximation and attachment of subsequent pairs of receptors and ligands. The process would be repeated many times until the pseudopods meet and fuse to form a phagocytic vacuole. Microfilaments were observed in the cytoplasm adjacent to bound capped lymphocytes and IgG-coated erythrocytes but were not seen around vacuoles containing completely ingested erythrocytes (Griffin et al., 1976).

The "zippering" theory implies that the position and density of binding sites on the phagocyte surface and ligands on the

particle must be of importance. It would thus explain experiments showing that the mere presence of binding sites for a particle may not be sufficient for its ingestion. Rabinovitch et al. (1975) showed that macrophages adherent to a surface coated with antigen-antibody complexes bound but did not ingest antibody-coated erythrocytes. Phagocytosis of latex particles or complement-coated red blood cells was unimpaired. Concanavalin A mediates binding but not phagocytosis of homologous erythrocytes by normal mouse macrophages whilst wheat-germ agglutinin can bring about both attachment and ingestion of the red blood cells (Goldman & Bursuker, 1976). Both lectins mediate binding and ingestion of yeast cells. It was shown that these differences could be related to differences in the number and density of lectin molecules bound to the macrophages, erythrocytes or yeast cells. These findings indicate the requirement for a critical density of receptors and ligands if binding is to lead to internalization. During the process of phagocytosis of IgG-coated particles the Fc-receptors appear to move laterally within the membrane to assemble where phagocytosis is taking place leaving the remainder of the cell surface devoid of such receptors (Romans et al., 1976). This redistribution of receptors might help to give a sufficient density of receptors for the phagocytosis of large particles.

Lateral movement of plasma membrane molecules in conjunction with phagocytosis appears to be partly non-random. The number of Fc-receptors and binding sites for concanavalin A on macrophages is reduced for a few hours following phagocytosis of latex particles (Schmidt & Douglas, 1972; Lutton, 1973) but membrane transport systems are not affected (Tsan & Berlin, 1971b). Microtubules may be responsible for this segregation of surface molecules (Ukena & Berlin, 1972).

9.2.2. The role of cellular contractile elements in phagocytosis. Reaven & Axline (1973) observed that microfilaments and microtubules accumulated in the cytoplasm next to the surface attached to a particle being phagocytosed by a macrophage suggesting that these cytoskeletal elements generate the cellular movement required in the process of phagocytosis. The contractile proteins of macrophages have since been studied in detail by Stossel and Hartwig (1976). These workers have isolated four contractile proteins from rabbit alveolar macrophages. They are actin, myosin, an actin-binding protein and a co-factor. The actin-binding protein causes actin to form a gel when combined with it. The co-factor is needed for the activation by actin of the Mg^{++} -dependent ATP-ase activity of macrophage myosin. When myosin and the co-factor are added with Mg^{++} and ATP to the actin gel the gel contracts. Electronmicrographs of these contracted gels show arrays of filaments. On the basis

of these observations Stossel (1976) has put forward a following hypothesis that when a particle makes contact with the macrophage membrane this causes activation of the actin-binding protein which promotes the polymerization of monomeric actin and cross-linking of the polymers into filaments. Myosin and the co-factor cause this actin gel to contract and thus lead to protrusion of a pseudopod alongside the particle. Stossel links his hypothesis with the "zippering" theory by suggesting that this process is repeated at sequential points of contact thus directing the movement of pseudopods around the particle. Cytochalasin B inhibits phagocytosis and was found also to inhibit the gelation of actin possibly by dissociating it from the actin-binding protein (Hartwig & Stossel, 1976). Colchicine has no effect on phagocytosis at concentrations that would interfere with microtubular function (Ukena & Berlin, 1972) but at very high concentrations, when it probably affects the plasma membrane rather than microtubules, it inhibited phagocytosis by mouse macrophages (Mimura & Asano, 1976). If, however, the cells were exposed to a low dose of colchicine or vinblastine with a sub-inhibitory concentration of cytochalasin D, phagocytosis was inhibited (Mimura & Asano, 1976). This suggests that a co-operative action of microtubules and microfilaments is important in phagocytosis.

9.2.3. Mechanisms of signal transmission in

phagocytosis. The mechanism of signal transmission from the surface of the plasma membrane to the cytoplasmic contractile elements is not known. It would seem not unlikely that ion fluxes were involved in a similar way to that suggested, with some experimental support, for chemotaxis. This has not been investigated but it has been reported that the active transport of K^+ into polymorphonuclear leucocytes is markedly depressed 10 minutes after the start of phagocytosis (Dunham et al., 1974). Michl et al. (1976a and b) have recently found that the glucose and mannose analogue 2-deoxy-D-glucose (2-DG) inhibits phagocytosis by mouse macrophages of IgG- or complement-coated particles without affecting their attachment. This inhibition was selective for phagocytosis mediated by the receptors for IgG or complement since phagocytosis of latex or zymosan particles was unaffected. This inhibitory effect could thus not be explained by blockage of the receptors or interference with the contractile response. It was also not linked to the decrease in intracellular levels of ATP caused by 2-DG. The authors suggested, therefore, that 2-DG interfered in some way with the steps that lead from binding to these specific receptors to the activation of contractile elements perhaps by disturbing glycosylation reactions.

9.2.4. Pinocytosis. Pinocytosis is a simpler process than phagocytosis. It has stricter metabolic requirements (Cohn, 1970b) but interaction with cell surface molecules is not required (Steinman et al., 1976). The pinocytic vesicles are formed by the protrusion and fusion of pseudopods.

10. INTRACELLULAR FATE OF INGESTED MATERIAL

The basic mechanisms of intracellular killing of micro-organisms and digestion of ingested material by macrophages are briefly outlined below. The changes in these functions that occur upon activation of macrophages will be dealt with in a later chapter.

10.1.1. Fusion of endocytic vesicles and lysosomes.

Following formation, the pinocytic vacuole moves into the perinuclear region to fuse with enzyme-containing primary lysosomes and forms secondary lysosomes (Cohn, 1970a). Phagocytic vesicles may also fuse with primary lysosomes but fusion with secondary lysosomes appears to be more common. This can be observed by incorporating a label into secondary lysosomes formed by pinocytosis and following the transfer of the label into the phagocytic vacuole (Jones & Hirsch, 1972; Goren et al., 1976). This process of fusion between endocytic and digestive vesicles can be inhibited by some substances such as concanavalin A (Edelson & Cohn, 1974) and suramin (Hart & Young, 1975). Virulent strains of Mycobacterium tuberculosis and viable Toxoplasma gondii also escape the action of lysosomal enzymes by this mechanism (Hart & Armstrong, 1974; Jones & Hirsch, 1972). Goren et al. (1976) have isolated a polyanionic sulphatide from virulent M. tuberculosis and shown that this substance prevents

fusion of phagosomes containing yeast cells with secondary lysosomes. Since the sulphatide entered secondary lysosomes it does not interfere with the membrane fusion of pinosomes and primary lysosomes. The authors speculate that recognition mechanisms operating between intracellular vesicles might be affected. Interestingly, suramin is also a polyanion and other polyanions may have similar effects (Goren et al., 1976). Specific antibodies enhance the fusion of secondary lysosomes with phagosomes containing toxoplasmas (Jones, 1974).

10.1.2. Lysosomal enzymes. Inside the secondary lysosome or phagolysosome the ingested material is exposed to the repertoire of lysosomal enzymes. These enzymes are all hydrolases and act in the acid environment of the lysosomes (Bowers, 1970). Lysosomal enzymes can digest a wide range of molecules including proteins, nucleic acids, polysaccharides and lipids (Cohn & Wiener, 1963; Bowers, 1970; Franson & Waite, 1973). Some of the more commonly studied enzymes include acid phosphatase, β -glucuronidase, β -galactosidase, the proteolytic cathepsins and lysozyme (Cohn & Wiener, 1963; Bowers, 1970; Braunsteiner & Schmalzl, 1970; Davies & Allison, 1976). Exposure to lysosomal hydrolases results in degradation of proteins into amino acids (Ehrenreich & Cohn, 1968; Cohn, 1970b). Lysosomes contain a number of glycolytic enzymes that cleave ingested saccharides. They lack, however, certain

enzymes required for the hydrolysis of some sugars including sucrose and some polysaccharides which therefore accumulate undigested in the lysosomes (Cohn, 1970b). Uptake of digestable material, such as aldehyde-treated erythrocytes or aggregated bovine gammaglobulin causes an increase in the level of lysosomal enzymes (Axline & Cohn, 1970). The presence of non-digestible material can in some cases stimulate the synthesis of lysosomal enzymes and may also induce their release into the extracellular medium. Particles or substances that have this effect are often associated with a chronic inflammatory reaction in vivo; they include asbestos, cell walls of group A Streptococci and carrageenan (Davies & Allison, 1976). Other non-digestible material, such as latex or carbon, appears to be completely inert, has no effect on the level of lysosomal enzymes and can remain in the body for a long time without eliciting any reaction (Axline & Cohn, 1970; Stuart, 1970).

10.1.3. Microbicidal mechanisms. Ingested living organisms are killed before digestion. The bactericidal mechanisms of mononuclear phagocytes are not as well studied as those of polymorphonuclear leucocytes and appear to be somewhat less efficient. In polymorphonuclear leucocytes the myeloperoxidase system is the most important mechanism (Klebanoff, 1975). Mature macrophages contain no demonstrable peroxidase but this enzyme is present in promonocytes and monocytes of some species

including man and mouse (Klebanoff & Hamon, 1975). It has been suggested that catalase might substitute for myeloperoxidase in some mononuclear phagocytes in a hydrogen peroxide-dependent microbicidal system (Klebanoff & Hamon, 1975) but there is not much supportive evidence. Macrophages also seem to lack the antibacterial cationic proteins found in the granules of polymorphonuclear leucocytes (Hirsch & Fedorko, 1970). During phagocytosis the uptake of oxygen by phagocytic cells is greatly increased (Karnovsky et al., 1970). In granulocytes this is accompanied by enhanced oxidation of glucose via the hexose-monophosphate shunt whilst in peritoneal macrophages there is equal stimulation of both this metabolic pathway and the Krebs cycle. These metabolic changes are linked to a markedly increased production of hydrogen peroxide in polymorphonuclear leucocytes. Hydrogen peroxide is essential for the myeloperoxidase system but can probably also have some microbicidal effect on its own (Klebanoff, 1975). Alveolar macrophages are capable of producing hydrogen peroxide but this is not much affected by phagocytosis (Rister & Baehner, 1977; Biggar & Sturgess, 1978). Other potentially microbicidal products of oxygen metabolism include the superoxide anion and hydroxyl radicals. Alveolar macrophages

do not produce as much of these radicals as polymorphonuclear leucocytes do and they also dispose of them more quickly (Rister & Baehner, 1977). On the whole oxygen-dependent microbicidal mechanisms appear, therefore, to be less important in macrophages as compared with granulocytes. However the killing of some bacteria by macrophages is impaired by lack of oxygen or inhibition of oxidative metabolism (Miller, 1971; Klebanoff & Hamon, 1975).

The oxygen-independent mechanisms of microbial killing and growth inhibition that may operate in the polymorphonuclear leucocyte include the acid environment of the phagolysosome, lysozyme, lactoferrin and granular cationic proteins (Klebanoff, 1975). As mentioned before macrophages do not possess these cationic proteins. Lactoferrin is also absent (Klebanoff & Hamon, 1975) but lysosomal acidity and lysozyme are present. Transferrin is produced and taken up by macrophages (Stecher & Thorbecke, 1967; MacSween & MacDonald, 1969) and its iron-chelating action might have a bacteriostatic effect.

10.1.4. Retention of undigested material. Another important difference between macrophages and polymorphonuclear leucocytes is that macrophages retain a small proportion of ingested material undigested, even of digestable material, whereas digestion by granulocytes appears to be complete (Cohn, 1967). This small amount of undegraded material can then be "presented" to lymphocytes as antigen (Unanue & Askonas,

1968; Unanue & Calderon, 1975; Roelants, 1977). Traces of antigen can persist for a long time inside certain types of macrophages and may be of importance for immunological memory (Unanue, 1972; Roelants, 1977).

11. INDUCTION OF THE SPECIFIC IMMUNE RESPONSE

It is not possible to discuss macrophage function without mentioning their role in the induction of the specific immune response. This important function of macrophages is, however, only marginally relevant to the present study and is summarized briefly in the following chapter.

11.1.1. Requirement for macrophages in the stimulation of lymphocytes. It is now well recognized that macrophages are required for the response of T-cells to antigens and mitogens (Rosenstreich & Oppenheim, 1976). T-cells need the help of macrophages in order to respond to antigen by proliferation (Rosenstreich & Oppenheim, 1976; Bach et al., 1970), lymphokine production (Yoshinaga & Waksman, 1973; Nelson & Leu, 1975; Epstein, 1976), generation of helper cells (Erb & Feldmann, 1975a and b) and cytotoxic cells (Wagner et al., 1972). The stimulation of cytotoxic cells seems to go through the intermediate step of the production of a factor by T helper cells (Plate, 1976) as occurs in the stimulation of B-cells by T-cell-dependent antigens. As expected the response by B-cells to T-cell-dependent antigens is also dependent on macrophages (Pierce & Kapp, 1976). Stimulation of B-cells with T-cell-independent antigens is far less sensitive to the depletion of macrophages (Pierce & Kapp, 1976) but there are experiments to show that a small number of macrophages is required even with

these antigens (Chused et al., 1976). The importance of macrophages in the stimulation of B-cells is also illustrated by several observations showing that macrophages can prevent tolerization of B-cells in certain situations (Braun & Lasky, 1967; Feldmann et al., 1974; Diener et al., 1976; Mosier, 1976).

11.1.2. Presentation of antigen on macrophages.

The action of macrophages in the stimulation of lymphocytes appears to be twofold. Firstly processing and presentation of soluble antigen and secondly secretion of activating factors. Following ingestion by macrophages most of the antigen is destroyed by digestion in a few hours but a small proportion, about 10%, is retained undigested for long periods (Unanue & Askonas, 1968; Nakano & Muramatsu, 1976). The retained antigen is exposed at the macrophages surface and also stored intracellularly. The surface-bound antigen is slowly released into the surrounding medium and can also be removed by trypsin. Following removal by trypsin antigen reappears on the surface by the process of exocytosis (Unanue & Calderon, 1975). Macrophages pulsed with antigen can stimulate specific antibody production by primed B-cells. This effect is significantly decreased by pretreatment of the macrophages with trypsin or antibody against the stimulating antigen (Unanue, 1978). Part of the antigen retained by macrophages appears therefore to be little changed from its

native form that is immunogenic for B-cells. In contrast there is some recent evidence that macrophage-bound antigen is partly changed into a form that can no longer be removed by trypsin or masked by specific antibody but is recognized by specifically immune T-cells (Ellner et al., 1977; Ben-Sasson et al., 1977; Thomas et al., 1978).

11.1.3. Stimulation of lymphocytes by soluble factors produced by macrophages. When the antigen is itself already cell-bound, such as allogeneic cells or tumour cells, the T-cells require far less help from macrophages in order to respond. It appears that the need for presentation of antigen on macrophages is redundant in those cases. Soluble macrophage-derived factors are, however, still required and may provide a necessary second signal for stimulation. These factors are non-specific in their action and can often be substituted by 2-mercaptoethanol in the medium (Rosenstreich & Oppenheim, 1976; Landolfo et al., 1977). Similarly this substance can replace macrophages in the antibody-producing response of spleen cells in vitro (Chen & Hirsch, 1972). Several macrophage-derived factors with a non-specific stimulating effect on lymphocyte function have been described. These include lymphocyte activating factor (LAF) which stimulates the proliferation of thymocytes and potentiates their responsiveness to mitogens (Gery & Waksman, 1972; Farr et al., 1977) and B-cell activating

factor (BAF) which promotes antibody production by T-cell-depleted spleen cells (Wood & Gaul, 1974; Wood et al., 1976). Lymphocyte activating factor (LAF) appears also to have an enhancing effect on either helper or suppressor T-cells and be capable of inducing antibody production by B-cells (Unanue, 1978; Koopman et al., 1978). These activities are, however, also found associated with molecules of different molecular weight (Unanue, 1978). None of these factors has been very clearly characterized and nothing is known about their activity in vivo. Production and secretion of LAF is enhanced by exposure to stimulated lymphocytes or their products (Meltzer & Oppenheim, 1977; Farr et al., 1977). This could represent a physiological stimulus and suggest a mechanism of positive feedback enhancement during the immune response. These non-specific factors listed above have usually been found to be equally active with syngeneic, allogeneic or even xenogeneic lymphocytes.

11.1.4. Models of the induction of the specific immune response. The mechanisms of initiation of the specific immune response, particularly antibody production, have been extensively studied in recent years. As more information has been gathered the picture has become increasingly complex and there are still many unanswered questions. It has become clear that several different cell types are involved and many currently proposed models include macrophages. Most such models are now based on the assumption that two distinct

signals are required for the stimulation of lymphocytes, viz. a specific signal from the antigen and a non-specific signal delivered by co-operating cells perhaps in the form of a soluble factor (see Bretscher, 1975). Several workers have described non-specific factors derived from T-cells that appear to provide a second signal for the activation of B-cells by antigen. These factors include so-called T-cell replacing factors (TRF) (Dutton et al., 1971; Schimpl & Wecker, 1975) and the allogeneic effect factor (AEF) of Armerding et al. (1974). The latter factor appears to contain Ia-antigenic determinants.

Antigen-specific T-cell factors that can replace T-cells in the stimulation of B-cells in vivo and in vitro have also been described. Thus Munro & Taussig (1975) obtained a T-cell-derived factor that was specific for the eliciting antigen and contained determinants coded for by the I-A sub-region of the H-2 complex (Taussig et al., 1975). The T-cell helper factor reported by Feldmann et al. (1974) is also antigen-specific but in contrast to the factor described by Munro & Taussig this factor appeared to be composed partly of an immunoglobulin but possessed no H-2-coded determinants. This factor was shown to stimulate the responses of B-cells in vitro to the relevant antigen only in the presence of macrophages.

The soluble T-cell factors mentioned above have usually been found to react with syngeneic as well as allogeneic B-cells.

In contrast, the experiments of Katz et al. (1975) using mixed cultures of T-helper cells and B-cells demonstrated a strict requirement for histocompatibility. It was found that identity at the I region was needed for successful collaboration. It was postulated that the I region contained cell interaction (CI) genes as well as immune response (Ir) genes. The product coded for by the CI genes would enable B-cells and T-cells to interact with each other.

The different models referred to above are not mutually exclusive and may all contain a germ of truth. There are some similarities and they may also partly explain different pathways that work together to achieve the specific stimulation of lymphocytes.

The induction of the specific immune response is obviously still an area of very active research. Some recent findings that may help to fill in the picture will be briefly mentioned here.

11.1.5. Recent findings on helper T-cells and their products. It has been found that helper T-cells can be divided into possibly three different types, viz. antigen-specific helper cells, non-specific helper cells (Tada et al., 1978) and so-called amplifier cells (Feldmann et al., 1977). The existence of both antigen-specific and non-specific helper T-cells would help to explain the differences between reported

T-cell factors. Howie & Feldmann (1977) have reported an antigen-specific T-cell helper factor that appears to be a hybrid between some of the previously described factors in that it possesses both Ia- and immunoglobulin determinants in addition to the antigen-binding site. It is thus possible that the factors described earlier were only fragments of the molecular complex constituting the complete helper factor.

11.1.6. Possible immune response gene control at macrophage level. Macrophages are heterogeneous in their capacity to stimulate lymphocyte responses and there is now evidence to suggest that this function may be largely performed by a sub-population of macrophages bearing Ia-antigens on the surface (Yamashita & Shevach, 1977; Niederhuber & Schreffler, 1977). This is particularly interesting in view of the suggestion that T-cells can only recognize antigen in some kind of conjunction with Ia-antigens on the macrophage-surface. (Schwartz et al., 1978). It has been suggested that the macrophage may select the antigenic determinant that is presented to T-cells and this selection would be under Ir-gene control at the level of the macrophage (Rosenthal et al., 1977; Rosenthal, 1978). Other evidence also suggests that immune response genes may govern the presentation of antigens by macrophages to lymphocytes or the interaction between these cells (Shevach & Rosenthal, 1973).

Recently Howie & Feldmann (1978) reported Ir-gene control of the interaction between mouse macrophages and B-cells mediated by a T-cell-derived helper factor. These authors also proposed that genetic restrictions operate at both T- and B-cell interactions with macrophages rather than directly between T-cells and B-cells. Some workers have now demonstrated that the secondary response of T-cells occurs only if the antigen is presented on macrophages of the same allotype as was used for priming but they may not have to be syngeneic (Thomas et al., 1977; Pierce & Kapp, 1978). Taken together these recent findings may help to solve some of the controversies about genetic constraints of the co-operation of B-cells and T-cells and their factors in vivo or in vitro and discrepancies between results based on primary or secondary immune responses.

11.1.7. Cluster formation between lymphocytes and macrophages. Interactions between macrophages and lymphocytes have been observed microscopically by Braendstrup & Werdelin (1977). It appears that only immune lymphocytes bind directly as "central" lymphocytes to macrophages pulsed with the relevant antigen. Other lymphocytes bind to the "central" lymphocyte and these "recruited" or "peripheral" lymphocytes can be either immune or non-immune. The non-immune "recruited" lymphocytes might possibly represent non-specific helper T-cells. B-cells do not seem to function as "central" lymphocytes and form only

a minor part of the "recruited" lymphocytes (Petri et al., 1978). This may be partly explained by the antigen used in these studies (purified protein derivative, PPD) or the interactions observed might reflect an early stage of the immune response before the stimulation of B-cells. Similar macrophage-lymphocyte clusters have also been demonstrated in the medullary sinuses of lymph nodes from recently immunized animals (Friess, 1977).

11.1.8. Regulation of the specific immune response.

The specific immune response appears to be regulated by specific and non-specific suppressor T-cells (see Möller, 1975 and Golub, 1977) as well as the specific antibody itself (Bystryn et al., 1970; Tew et al., 1976). There is also some evidence that macrophages may have a regulatory function by non-specifically inhibiting lymphocyte responses. It is well known that excessive numbers of macrophages (more than about 10% of the total cell population) have an inhibitory rather than stimulatory effect on B- and T-cell responses (Harris, 1965; Hoffmann, 1970; Lenai & Feldman, 1971). Similarly supernatants from macrophages stimulate mitogen-induced proliferation of T-cells only up to a certain concentration, higher concentrations are inhibitory (Yoshinaga et al., 1975). Baird & Kaplan (1977a and b) have shown that the effect of macrophages and their supernatants is

to inhibit the formation of lymphoblasts rather than to kill newly formed blasts. Administration of some macrophage-activating agents, such as BCG and C. parvum, is associated with a depression of T-cell responses and this effect is dependent on macrophages (Mitchell et al., 1973; Klimpel & Henney, 1978; Scott, 1972). Spleen cells from mice undergoing a graft-versus-host reaction also show a macrophage-dependent depression of T-cell helper function in antibody production (Elie & Lapp, 1977). Activated macrophages or their supernatants have been found to have a more pronounced cytostatic effect on lymphocytes as compared with normal macrophages (Nelson, 1973 & 1976; Keller, 1976). As macrophages can be activated by lymphokines (see following chapter) this might provide a negative feedback mechanism for controlling immune responses. Little is known about the mechanisms by which macrophages inhibit lymphocyte responses but some of the produced factors that have been suggested for such a function include arginase, polyamine oxidase, complement cleavage products and prostaglandins (Allison, 1978; Webb & Nowowiejski, 1977). One report based on studies on five human patients with recurrent fungal infections suggests that macrophages may exert their suppressive effect on T-cell responses by acting through a subpopulation of regulator T-cells (Stobo, 1977).

11.1.9. Concluding remarks. It is too early to come to an overall conclusion about the mechanisms of the induction of the specific immune response. Present evidence suggests initial interaction of T-cells with modified antigen in conjunction with Ia-antigens on the surface of macrophages. The stimulation of T-cells is then also promoted by macrophage-derived soluble factors. The T-cells appear to differentiate into specific and non-specific helper T-cells releasing specific and non-specific factors. The stimulation of antibody production by B-cells is then achieved by an interaction between B-cells, the T-cell-derived factors and native antigen presented on macrophages. These processes appear to be regulated by suppressor T-cells, specific antibody and inhibitory factors released by macrophages.

12. STIMULATION AND ACTIVATION OF MACROPHAGES

The present study is partly concerned with the mechanisms of macrophage activation. The following chapter gives a definition of stimulated and activated macrophages, describes methods of stimulation and activation as well as the morphological and functional characteristics of stimulated and activated macrophages. Possible mechanisms of activation will be discussed in a separate chapter.

12.1 Definition of the activated and stimulated macrophage.

12.1.1. Activated macrophages. The terms "stimulated" and "activated" macrophages are used rather arbitrarily and there is no commonly agreed definition. Some workers have, therefore, stressed the need to use "activated macrophages" only within a strictly defined context with reference to a specific stimulus and the response to it (Evans & Alexander, 1976; Davies & Allison, 1976). Mackaness (1962, 1970a) described the functional changes that occur in macrophages during an in vivo infection with intracellular bacterial parasites. Macrophages of animals that had recently recovered from such an infection showed a greatly increased capacity to ingest and destroy a wide range of intracellular bacterial parasites and Mackaness called them "activated". Subsequently macrophages with cytotoxic activity against tumour cells were termed "activated" (Alexander & Evans, 1971; Hibbs et al., 1972). Normal macrophages or

macrophages elicited by the acute inflammatory reaction to irritants such as casein or thioglycollate do not have these functional capacities. These two functional characteristics can both be induced by exposure of macrophages to immune lymphocytes or lymphokines (see David & Remold, 1976) and can be seen as an expression of the role of macrophages as effector cells in cell-mediated immunity. The ability to non-specifically inhibit the growth of or kill bacteria or protozoa that are intracellular parasites or to exert a cytotoxic effect against nucleated cells can therefore be regarded as major criteria of macrophage activation. Macrophages that are activated by these criteria show several other morphological and functional differences from normal macrophages. Morphologically, activated macrophages are markedly different from normal macrophages but macrophages elicited by irritants can look quite similar (Blanden et al., 1969; Hirsch & Fedorko, 1970). Macrophages that have been shown to be cytotoxic have also been found to respond more vigorously to a chemotactic stimulus (Meltzer et al., 1975) and produce more lymphocyte activating factor (LAF) (Meltzer & Oppenheim, 1977) than normal or irritant-elicited macrophages. Recently Kaplan & Morhanakumar (1977) have demonstrated the appearance of a new surface antigen on cytotoxic macrophages. Stimuli such as lymphokines and bacterial lipopolysaccharides, that can render macrophages cytotoxic and enhance their antimicrobial activity, also induce increased

incorporation of glucosamine by macrophages (Hammond & Dvorak, 1972) and the function of the C3-receptor is changed in such a way that binding leads to internalization (Mørland & Kaplan, 1977). Lymphokines as well as many substances associated with chronic inflammation cause an increase in synthesis of lysosomal enzymes and induce their release (Davies & Allison, 1976; Pantalone & Page, 1977). The functional characteristics just mentioned, increased chemotaxis, increased LAF-production, new surface antigen, increased incorporation of glucosamine, phagocytosis via the C3-receptor and secretion of lysosomal enzymes have all been suggested or used as working criteria for macrophage activation. They may be seen as minor criteria of activation. The correlation between the major and minor criteria of macrophage activation has not been well enough established and it can therefore not be safely concluded that macrophages that fulfil some of the minor criteria will also be antimicrobial against intracellular parasites and/or cytotoxic. The two major criteria are often present together (Ruskin et al., 1969; Hibbs et al., 1972) but this may not always be the case as demonstrated recently by Wing et al. (1977). Their observations may reflect either different time scales for development and loss of different functional capacities or alternatively the existence of different macrophage sub-populations. This emphasises again that macrophages are a heterogeneous population which makes generalizations difficult.

In the present work only macrophages fulfilling one or both of the major criteria will be called activated, otherwise the functional response will be stated.

12.1.2. Stimulated macrophages. The macrophages that appear in the peritoneal cavity following injection of certain agents such as casein or thioglycollate are different from both normal and activated macrophages. Macrophages with similar characteristics arise in vitro following exposure to heterologous serum (Gohn & Benson, 1965a). These macrophages are often morphologically very similar to activated macrophages and can show some of the metabolic characteristics of activated macrophages. The activity of many enzyme systems may be markedly changed from normal (Karnovsky et al., 1975a). Several different names have been used for this type of macrophages, including elicited, induced and stimulated macrophages. Here they will be called stimulated macrophages. Stimulated macrophages are not cytotoxic or antimicrobial against intracellular parasites. One important difference between normal and stimulated macrophages is that the latter can be activated in vitro by agents that would normally only activate macrophages when administered in vivo (Christie & Bomford, 1975).

12.2 Stimulating and activating agents.

12.2.1. Stimulating agents *in vivo*. Stimulating or inducing agents injected intraperitoneally are used mainly to increase the otherwise low yield of macrophages from species such as guinea pigs and rats but are also frequently used for mice. They give rise to a local inflammatory response that will include polymorphonuclear leucocytes in the early stages. The more commonly used agents include mineral oil, thioglycollate broth, proteose peptone broth, casein and glycogen. Some of these agents are not readily degradable and remain in the cells (Stuart *et al.*, 1973). The main disadvantage of their use is that the macrophages obtained in this way are not normal and respond to further stimulation *in vitro* in a different way from normal cells (Christie & Bomford, 1975; Davies *et al.*, 1977).

12.2.2. Stimulating agents *in vitro*. Heterologous serum added *in vitro* causes changes in macrophage morphology and metabolism akin to those brought about by stimulating agents *in vivo* (Cohn & Benson, 1965a). This appears to be mediated by the immunoglobulin fraction of the serum and does not occur when foetal serum is used (Cohn & Parks, 1967). Several other macromolecules particularly those that are anionic such as dextran sulphate, hyaluronic acid and DNA also stimulate the pinocytic activity of macrophages in a similar

way as heterologous serum (Cohn, 1970b) and lectins have recently also been shown to have this effect (Goldman et al., 1976).

Phagocytosis stimulates the metabolism of macrophages but the nature and extent of such stimulation depends on the particle being ingested and whether or not the macrophages had been previously stimulated. Thus phagocytosis of starch granules by stimulated macrophages leads to an increase of glucose oxidation (Oren et al., 1963). Ingestion of starch or latex has, however, no effect on the level of lysosomal enzymes which is raised following the phagocytosis of digestible material such as aldehyde-fixed erythrocytes or aggregated gammaglobulin (Cohn, 1970a). Phagocytosis of inert particles can also induce secretion by macrophages of neutral proteinases and LAF particularly if the macrophages have been stimulated in vivo (Davies et al., 1977; Unanue et al., 1976).

12.2.3. Lymphocyte-dependent activating agents.

Activation of macrophages classically occurs in animals that are infected with intracellular pathogens. These pathogens include bacteria such as Mycobacteria, Listeria and Brucella (Mackaness, 1970b; North, 1969; Ruskin et al., 1969; Hibbs et al., 1972) and protozoa such as Toxoplasma, Trypanosoma and Besnoitia (Ruskin et al., 1969; Hibbs et al., 1972; Remington et al., 1972; McLeod & Remington, 1977). Chronic infestation of mice with Trichinella spiralis also leads to the generation of activated macrophages (Wing et al., 1977). In these

infections the activation of macrophages is dependent on the presence of lymphocytes (Mackanness, 1964 & 1970b; Lane & Unanue, 1972; North, 1973). The lymphocytes respond to antigens of the infecting agent in a specific way and activate the macrophages to become non-specifically capable of killing a wide range of intracellular parasites. This activated state disappears as the animals recover from the infection but can be rapidly recalled by secondary challenge with the same antigen (Mackanness, 1970b). Macrophages can also be activated in vitro by exposure to sensitized lymphocytes and the relevant antigen (Simon & Sheagren, 1971; Krahenbuhl & Remington, 1971; Simon & Sheagren, 1972). The lymphocytes responsible for macrophage activation in infections with intracellular parasites have been shown to be T-lymphocytes (Lane & Unanue, 1972; North, 1969; Krahenbuhl et al., 1973). There are, however, cases when exposure of macrophages to micro-organisms seems to result in activation in the absence of T-lymphocytes. Thus it has been shown that congenitally athymic "nude" mice respond to infection with Listeria and Brucella and their macrophages become even more efficient at killing these bacteria than the macrophages from normal infected mice (Cheers & Waller, 1975). Activation by a killed vaccine of C. parvum appears to be T-cell-independent in vivo but T-cell-dependent in vitro for normal macrophages (Ghaffar et al., 1975; Ghaffar & Cullen, 1976; Šljivić & Watson, 1977). Macrophages that have been

stimulated in vivo can, however, be activated by C. parvum in vitro but in that case bacterial strains that are not normally associated with macrophage activation are equally effective (Christie & Bomford, 1975). It has been speculated that in these cases of apparent T-cell-independent macrophage activation by micro-organisms the macrophages may already have been stimulated by bacterial products derived from altered or increased gut flora caused by the T-cell deficiency (Cheers & Waller, 1975). Alternatively B-cells may have taken the role normally fulfilled by T-cells. This is supported by the finding that B-cells can also release non-specific lymphokines, a function normally attributed to T-lymphocytes (Yoshida et al., 1973; Rocklin et al., 1974). B-cells also seem to be required for the stimulation in vitro by bacterial lipopolysaccharide of glucosamine uptake by guinea pig macrophages (Wilton et al., 1975) although activation of mouse macrophages by the same agent appears to occur without the intervention of T- or B-lymphocytes (Alexander & Evans, 1971; Evans & Alexander, 1976).

12.2.4. Lymphocyte-independent activating agents.

Several agents have now been shown to activate macrophages directly without a requirement for lymphocytes. Many polyanions have this property, including double-stranded RNA, bacterial endotoxin and pyran copolymer (Evans & Alexander, 1976; Baird & Kaplan, 1975; Schultz et al., 1977a). Prolonged culture

with some bacteria or their derivatives such as BCG, mycobacterial extracts and cell walls of group A Streptococci also results in activation of macrophages (Bruley-Rosset et al., 1976; Schwab & Smialowicz, 1975). These agents have all been shown to activate purified ($\geq 95\%$) cultures of macrophages in vitro. It can of course be argued that the low proportion of lymphocytes remaining in these cultures is sufficient to mediate activation but the activation in vitro by lymphocyte-dependent agents has been found to require a relatively large number of lymphocytes (Krahenbuhl & Remington, 1971; Christie & Bomford, 1975; Wilton et al., 1975).

12.2.5. Soluble mediators of activation. In in vitro models of macrophage activation whole lymphocytes can often be replaced by supernatants derived from lymphocytes stimulated by antigens or mitogens. Activation by lymphokines has been most extensively studied using guinea pig macrophages and appears to be mediated by a molecule similar to or possibly identical with MIF (David & Remold, 1976). As the molecular structure of these lymphokines has not been identified in detail they are normally referred to by their function. Thus lymphocyte supernatant factors that activate macrophages are called macrophage activating factors (MAF). These factors are not antigen-specific and appear to show no species specificity (David & Remold, 1976; Lohmann-Matthes, 1976; Pantalone & Page, 1977). From recent studies with mouse lymphokines and

macrophages it appears that in this species more than one lymphokine may act together in the metabolic stimulation of macrophages (Lazdins et al., 1978). Certain lymphokines appear to be antigen-specific. The best known of these is the specific macrophage arming factor (SMAF) described by Evans & Alexander (1972). This factor binds to macrophages and makes them cytotoxic for the specific target cells. Incubation with the specific antigen subsequently leads to non-specific activation of the macrophages. Specificity was also demonstrated in the experiments of Borges & Johnson (1975) who found that normal human monocytes could be activated to kill Toxoplasma gondii by exposure in vitro to the products of Toxoplasma-immune lymphocytes but not lymphocytes stimulated by different antigens or mitogens. Certain differences have been observed between activation by lymphokines and that brought about by antigen-stimulated lymphocytes themselves. Thus the time course is longer for activation with lymphokines and the macrophages may become only bacteriostatic rather than bactericidal (David & Remold, 1976). Direct contact with stimulated lymphocytes is a more potent stimulus than exposure to lymphokines for enzyme production by macrophages (Ando et al., 1976) and macrophages can be "armed" by close contact with sensitized lymphocytes alone in the absence of antigen (Evans & Alexander, 1976). It is possible that lymphocytes secrete additional labile factors or that the lymphokines can be more effectively presented

to macrophages by direct contact with lymphocytes. Although macrophage "arming" by sensitized lymphocytes in the absence of antigen was equally effective in syngeneic and allogeneic systems (Evans & Alexander, 1976) a requirement for histocompatibility has recently been demonstrated for the stimulation by immune lymphocytes of glucose oxidation (Riisgaard et al., 1978) and secretion of LAF (Farr et al., 1977). There is thus evidence for macrophage activation both by direct contact with immune lymphocytes and mediated by soluble lymphokines which can be specific for an antigen although they usually appear to be non-specific.

The specific macrophage arming factor, SMAF, appears to be a T-lymphocyte product and these cells are also the main source of non-specific lymphokines although these can also be produced by B-cells as mentioned above. B-cell-derived immunoglobulins may also play a role in macrophage activation. Cytophilic antibody may "arm" macrophages in a way similar to the action of SMAF and can induce cytotoxicity upon contact with the antigen. Immune complexes and heat-aggregated serum as well as anti-immunoglobulins have been shown to render macrophages non-specifically cytotoxic (Evans, 1975; Evans & Alexander, 1976). The ability to "arm" or activate macrophages may be the function of some classes and sub-classes of immunoglobulins and full activation probably only occurs when the immunoglobulin is bound to antigen or aggregated. Thus only the IgG2b subclass

but not IgG2a or IgM mediates cytolysis of chicken erythrocytes by mouse macrophages (Walker, 1977). IgG2b only binds to macrophages when aggregated or bound to antigen. Rat macrophages become capable of killing schistosomes following exposure to serum from rats infected with Schistosoma mansoni which contains antigen. Activation was shown to depend upon the IgE present in the serum (Capron et al., 1977).

Two other non-specific mediators of macrophage activation have recently been described, C3b (Schorlemmer & Allison, 1976; Schorlemmer et al., 1977a; Ferluga et al., 1978) and interferon (Schultz et al., 1977). Both have been shown to render macrophages cytotoxic after incubation in vitro. C3b and interferon could function as mediators of the effect of some lymphocyte-independent activating agents. Thus streptococcal cell walls, BCG and several polyanions activate the alternative pathway of complement (Schorlemmer et al., 1977b) and polyanions induce the secretion of interferon (Schultz et al., 1977b). Macrophages can themselves produce factors that cleave C3 (Schorlemmer & Allison, 1976) as well as interferon (Schultz et al., 1977b) when stimulated and this might provide a positive feedback mechanism, augmenting and maintaining the activated state.

12.3 Morphological and metabolic changes following stimulation and activation.

12.3.1. Morphology, adherence and phagocytosis.

Stimulated and activated macrophages are larger than normal macrophages and show more rapid and extensive spreading on a surface. Pseudopodia and motile membrane ruffles are more prominent as well as cytoplasmic granules and vacuoles (Blanden et al., 1969; Hirsch & Fedorko, 1970; Carr & Carr, 1970; Nath et al., 1973). Macrophages that have been stimulated in vivo or activated in vivo or in vitro show increased surface adherence and pinocytosis as well as accelerated phagocytosis of some particles but not others (Nathan et al., 1971; Keller et al., 1974; Karnovsky et al., 1975b; Edelson & Erbs, 1978).

12.3.2. Uptake of nutrients and metabolism.

Stimulation and activation of macrophages leads to several changes in their metabolism. The carrier-mediated transport of glucose across the plasma membrane is enhanced in macrophages that have been treated with lymphokines or following phagocytosis. Transport of leucine, however, appears only to be stimulated by lymphokines but not by phagocytosis (Bonventre et al., 1977). The oxidation of glucose via the hexose monophosphate shunt is accelerated in stimulated as well as activated macrophages (Karnovsky et al., 1975b; Poulter & Turk, 1975a & b) but the Krebs-cycle appears to be stimulated only in activated macrophages

(Karnovsky et al., 1975b). Karnovsky et al. (1975b) detected no increase in the uptake of oxygen in activated or stimulated macrophages unless they were phagocytosing whilst normal macrophages did not respond to phagocytosis in this way. The production of superoxide was depressed in both activated and stimulated macrophages (Karnovsky et al., 1975a). The RNA-content of macrophages is increased following exposure to stimulating and activating agents (Stadler & de Weck, 1978). Protein synthesis in guinea pig macrophages was found to be unaffected by treatment with lymphokines in vitro (Nathan et al., 1971) but in macrophages from rats injected with proteose-peptone an increase in protein synthesis was shown to correlate closely with the expression of cytotoxicity (Keller et al., 1974).

The uptake of glucosamine, mainly into the Golgi region, is enhanced following a few days of culture with sensitized lymphocytes and antigen (Hammond & Dvorak, 1972) or bacterial endotoxin (Wilton et al., 1975).

12.3.3. Membrane-bound and cytoplasmic enzymes.

The activity of several plasma membrane enzymes has been studied in stimulated and activated macrophages. The activity of 5'-nucleotidase (AMP-ase) is markedly decreased in stimulated macrophages and to a lesser extent in activated macrophages (Karnovsky et al., 1975a; Edelson & Cohn, 1976; Edelson & Erbs, 1978). This has been found to be caused by accelerated

breakdown of the enzyme (Edelson & Cohn, 1976). Karnovsky et al. (1975a) found enhanced activity of ATP-ase in activated macrophages whilst the activity of p-nitrophenylphosphatase was slightly increased in activated macrophages and more markedly so in stimulated macrophages. Exposure to lymphokines leads to stimulation of adenylcyclase in guinea pig macrophages (Remold-O'Donnell & Remold, 1974) but this is not associated with an increase in the intracellular levels of cyclic AMP (Higgins et al., 1976; Pick, 1977). Aminopeptidase (leucine-naphthylamidase) is found in increased amount in the plasma membrane of stimulated macrophages but not macrophages that have been exposed to the activating agent bacterial lipopolysaccharide (Wachsmuth & Stoye, 1977). This enzyme along with the cytoplasmic enzyme peroxidase appears to be a characteristic of young mononuclear phagocytes that is lost with further maturation (Wachsmuth & Staber, 1977; van Furth et al., 1970). Peroxidase is not found in activated macrophages but is present in stimulated macrophages (Karnovsky et al., 1975a). This enzyme might play a role in bacterial killing as mentioned in an earlier chapter but clearly other mechanisms are more important in activated macrophages. Another cytoplasmic enzyme, lactic dehydrogenase, is found in increased amounts in macrophages that have been treated with lymphokines (Remold & Mednis, 1972) or bacterial endotoxin (Davies et al., 1974) and

this is probably linked to the enhanced metabolism of glucose. Stimulated and activated macrophages thus show different patterns of altered enzyme activity. Stimulated macrophages tend to express enzymes that are characteristically found in young mononuclear phagocytes indicating that these cells have newly arrived in the inflammatory exudate. Little is known about the functional significance of the changed enzyme activity in most cases.

12.3.4. Lysosomal enzymes. The reported effects of stimulation or activation on the levels of lysosomal enzymes are somewhat variable. Bar-Eli & Gallily (1975) found that different stimulating agents caused different patterns of increase in the levels of 4 lysosomal enzymes in mouse macrophages. Activated rat macrophages show increased activity of various lysosomal enzymes (Keller et al., 1974). Macrophages from mice treated with BCG have increased levels of some lysosomal enzymes but the amount of lysosomal phospholipases is lower than in normal macrophages (Franson & Waite, 1973). In contrast the total concentration of several lysosomal enzymes was found to be lowered following treatment of oil-induced guinea pig macrophages with lymphokines (Remold & Mednis, 1972). It is known that several stimuli lead to the selective release of lysosomal enzymes from macrophages into the surrounding medium

(Davies & Allison, 1976) but this could not explain the findings of Remold & Mednis (1972) as the fall in intracellular enzyme levels was not correlated with a rise in extracellular levels. These conflicting results might possibly be explained by variations between animal species or differences in the response of normal and stimulated cells to activating agents.

12.3.5. Cell surface receptors. The activity of cell surface receptors is also changed following stimulation and activation. The number of Fc-receptors has been found to be increased in activated and stimulated macrophages (Arend & Mannik, 1973; Rhodes, 1975a) and binding to the complement receptor leads to phagocytosis only if the macrophages are stimulated or activated (Bianco et al., 1975; Morland & Kaplan, 1977) which may also reflect a greater number of receptors or different distribution. The response to migration inhibition factor is increased in stimulated macrophages (Leu et al., 1977). Activated macrophages bind more effectively to tumour cells (Piessens, 1978). Recently activated macrophages have been found to express a new antigen on their surface. This is not found on normal or stimulated cells and its expression appears to be quite strictly confined to cytotoxic macrophages (Kaplan & Morhanakumar, 1977; Kaplan et al., 1978).

12.4 Secretory functions of stimulated and activated macrophages.

Normal macrophages synthesize and secrete plasma proteins such as transferrin and several complement factors as mentioned in an earlier chapter. Lysozyme is also constantly secreted by macrophages without any stimulation. Thioglycollate-induced macrophages show some increase in secretion of lysozyme but phagocytosis has no effect (Gordon *et al.*, 1974a). Factors that influence lymphocyte responses and colony stimulating factor have also been found to be secreted by unstimulated macrophages but only in small quantities (Gery & Wiener, 1975; Meltzer & Oppenheim, 1977; Wood & Cameron, 1976; Eaves & Bruce, 1974) and there is a small constant release of prostaglandins (Humes *et al.*, 1977). In addition Melsom *et al.* (1974) have detected a labile factor produced by unstimulated macrophages *in vitro* that is cytotoxic for syngeneic and allogeneic erythrocytes.

Stimulated and activated macrophages are very active secretory cells and synthesize and secrete substances that are not found in normal macrophages or only synthesised without being secreted. The range of substances secreted depends to some extent on the nature of the stimulus.

12.4.1. Factors that act on lymphocytes. Mouse macrophages secrete increased quantities of LAF following

exposure in vitro to bacterial endotoxin, concanavalin A or lymphokines (Gery & Wiener, 1975; Meltzer & Oppenheim, 1977). Activation in vivo by BCG, pyran or bacterial endotoxin has the same effect (Meltzer & Oppenheim, 1977). Unanue et al. (1976) found that stimulation with thioglycollate had no effect on the production of LAF by mouse macrophages. They showed, however, that phagocytosis of various particles, including latex and antibody-coated erythrocytes, was sufficient to induce the release of LAF from normal or peptone-induced macrophages whilst thioglycollate-induced macrophages did not respond in this way. The findings of Unanue et al. (1976) would not imply a close relationship between LAF-production and other functions of activated macrophages. The release of LAF in these experiments was short-lived when using either normal macrophages, stimulated by phagocytosis or activated macrophages from mice infected with Listeria thus indicating that little or no resynthesis was taking place during culture. Synthesis could be induced by incubating normal macrophages or macrophages from Listeria-infected mice with antigen-stimulated lymphocytes. It appears, therefore, that increased synthesis of LAF is an attribute of activated macrophages only whilst release of preformed LAF can be induced by stimuli such as phagocytosis. Secretion of B-lymphocyte activating factor (BAF) by human monocytes in culture can be stimulated by bacterial endotoxin and phytohaemagglutinin but not latex particles or immune complexes (Wood & Cameron, 1976).

12.4.2. Lysosomal enzymes. Several agents that are associated with chronic inflammation have been shown to cause a selective release of lysosomal acid hydrolases. These stimuli include cell walls of group A streptococci, dental plaque, carrageenan, mouldy hay dust and asbestos (Davies & Allison, 1976; Schorlemmer et al., 1977c). Immune complexes also have this effect (Cardella et al., 1974) as well as polyanions and zymosan (Schorlemmer et al., 1977b). This release is often not accompanied by an increase in synthesis (Davies et al., 1974; Schorlemmer et al., 1977b). Lymphokines induce the release of lysosomal enzymes after several hours of contact and after a longer lag period resynthesis is stimulated (Pantalone & Page, 1976).

12.4.3. Neutral proteinases. The secretion by macrophages of non-lysosomal neutral proteinases such as plasminogen activator, collagenase and elastase has been studied by several workers recently (see Gordon, 1976). These enzymes are not synthesised or secreted by normal macrophages (Werb & Gordon, 1975a & b). Macrophages from mice stimulated with thioglycollate in vivo synthesise and secrete these enzymes over long periods in culture (Werb & Gordon, 1975a & b; Unkeless et al., 1974). Phagocytosis of particles such as latex has also been found to induce the secretion of small amounts of collagenase and elastase (Werb & Gordon, 1975a & b).

Macrophages harvested after treatment in vivo with bacterial endotoxin did not secrete plasminogen activator but could be stimulated to release this enzyme after phagocytosis in vitro of various particles thus implying that stimulation can occur in two stages (Gordon et al., 1974b). Similarly exposure to bacterial endotoxin in vitro can stimulate the release of collagenase by oil-induced guinea pig macrophages (Wahl et al., 1974). It has been shown that lymphokines can stimulate the secretion of plasminogen activator (Vassalli & Reich, 1977) and collagenase (Wahl et al., 1975; Klimetzek & Sorg, 1977) and mice infected with Trypanosoma cruzi or BCG secrete large amounts of plasminogen activator. The macrophages from these infected mice could also kill T. cruzi organisms (Nogueira et al., 1977). Interestingly many of the stimuli that cause release of lysosomal enzymes have no effect on the neutral proteinases (Davies & Allison, 1976). The synthesis and secretion of neutral proteinases appears thus to be a property of stimulated and activated macrophages and is promoted by phagocytosis. The release of these enzymes is of potential importance in tissue destructive disease processes such as emphysema and rheumatoid arthritis (Gordon, 1976; White et al., 1977).

12.4.4. Prostaglandins. Macrophages are a source of prostaglandins and their release is greatly increased following phagocytosis of zymosan particles in vitro (Humes et al.,

1977); stimulation by thioglycollate in vivo or phagocytosis in vitro of latex particles had no effect (Bonney et al., 1977; Humes et al., 1977). Activation in vivo by C. parvum (Farzad et al., 1977) or in vitro by lymphokines (Gordon et al., 1976) also stimulates the release of prostaglandins. Prostaglandin-release by macrophages may be important in the regulations of lymphokine-secretion (Gordon et al., 1976) and myelopoiesis (Kurland et al., 1978).

12.4.5. Cytotoxic and bacteriostatic factors.

Activated macrophages have been shown to release cytotoxic and bacteriostatic factors that appear to be fairly labile under ordinary culture conditions and are inhibited in their action by the presence of serum in the medium (Currie & Basham, 1975; Middlebrook et al., 1974).

12.4.6. Other secretory products.

Recently a macrophage-derived factor has been described that induces neo-vascularization and could thus play a role in stimulating vascular proliferation that occurs in wound healing and chronic inflammation. This factor was not released by normal macrophages but secretion could be induced by stimulating the macrophages with oil or thioglycollate in vivo or latex particles in vitro (Polverini et al., 1977).

Macrophages can be induced to secrete pyrogen by exposure in vitro to endotoxin or heat-killed Staph. albus (Bodel, 1974; Dinarello et al., 1974) and they produce interferons both in response to viruses and non-viral stimuli such as endotoxin or BCG in vivo and lymphokines in vitro (Smith & Wagner, 1967; Neumann & Sorg, 1977). Macrophages that have been exposed to lymphokines in vitro secrete increased amounts of the second component of complement (Littman & Ruddy, 1977). Mouse macrophages have also been shown to secrete factor B of the alternative pathway of complement activation. Thioglycollate-stimulated macrophages secreted less factor B than normal cells but responded more vigorously to a phagocytic stimulus by enhanced secretion (Bentley et al., 1977). Treatment of human monocytes in vitro with physiological concentrations of hydrocortisone has been shown to induce the release of a substance that is chemotactic for polymorphonuclear leucocytes (Stevenson, 1974).

12.5 Antimicrobial and cytotoxic activity of activated macrophages.

12.5.1. Antimicrobial activity against intracellular parasites. Normal macrophages can phagocytose and kill pyogenic bacteria such as Staphylococci and E. coli (van Zwet et al., 1975) but bacterial and protozoal intracellular parasites survive and multiply inside normal macrophages. By the definition used here activated macrophages can limit the growth of these intracellular parasites or kill them whilst stimulated

macrophages do not have this capacity. The growth inhibition or killing can be measured after in vivo infection by culturing bacteria from infected organs (Mackanness, 1962 & 1964) or in in vitro experiments by incubating macrophages that have phagocytosed organisms and examining the cells microscopically for the presence and growth of micro-organisms (Krahenbuhl & Remington, 1971) or culturing measured amounts of macrophage lysates on bacterial culture media (Fowles et al., 1973). In the case of protozoa the uptake of radiolabelled uridine can be measured (Remington et al., 1972). The intracellular micro-organisms that can be killed by activated macrophages include the bacteria Listeria monocytogenes, Brucella, Mycobacteria (Bacillus Calmette-Guérin) and Salmonellae (Mackanness, 1962, 1964 & 1970a) and the protozoa Toxoplasma gondii (Remington et al., 1972), Trypanosoma cruzi (Nogueira et al., 1977) and Leishmania (Farah et al., 1975; Behin et al., 1975). Activated macrophages are important in the recovery from these infections and the rapid regeneration of activated macrophages on secondary challenge mediated by immune T-lymphocytes provides an efficient mechanism of immunity (Mackanness, 1970b; North, 1970).

This effector function of activated macrophages is usually regarded as non-specific (Mackanness, 1970b). In some cases there appears, however, to be some degree of specificity particularly in some of the protozoal infections. Thus vaccination with killed C. parvum was found to protect mice

against subsequent challenge with Brucella (Adlam et al., 1972), Listeria or avirulent Toxoplasma (Swartzberg et al., 1975).

The protection against Listeria or Toxoplasma was, however, of a lower degree than if the mice had been previously infected by the homologous organism and there was no increase in the resistance against virulent Toxoplasma following treatment with C. parvum (Swartzberg et al., 1975). Peritoneal exudate macrophages from mice treated with C. parvum were capable of killing Toxoplasma in vitro (Swartzberg et al., 1975; McLeod et al., 1977) thus illustrating that the ability of macrophages to kill a micro-organism in vitro may not imply resistance against infection in vivo. In these experiments the route of administration of C. parvum appeared to be important perhaps indicating activation of different populations of macrophages at different anatomical sites. Macrophages from BCG-treated mice are not as efficient at killing T. cruzi as macrophages from animals infected with T. cruzi (Nogueira et al., 1977). Infection with Besnoitia increases the resistance of mice to Toxoplasma but not to the same degree as infection with the homologous organism (McLeod & Remington, 1977). Macrophages from mice with either of these infections killed Toxoplasma organisms in vitro equally effectively. Some of these observations may be explained by the need for prolonged release of antigens from living organisms for successful immunization against these protozoa (Nogueira et al., 1977) and the maintenance

of activated macrophages by the presence of stimulated T-cells (McLeod & Remington, 1977). Chemotactic factors and MIF released by immune T-cells might be important in attracting macrophages to the sites of infection and keeping them there.

The mechanisms of killing or growth inhibition of intracellular parasites by activated macrophages are largely unknown. It has not been established whether activated macrophages produce different kinds of bactericidal substances from those found in normal macrophages or whether the difference is a quantitative one. The secretion of a labile factor that can kill Listeria monocytogenes by activated macrophages has been reported (Middlebrook et al., 1974). This factor could not kill Salmonellae. The production of superoxide anions is decreased rather than increased in activated macrophages and therefore not likely to be important in the enhanced microbicidal capacity of activated macrophages (Karnovsky et al., 1975a). It was mentioned in an earlier chapter that very little is known about the bactericidal mechanisms of mononuclear phagocytes as compared with polymorphonuclear leucocytes (see 10.1.3.).

12.5.2. Antiviral activity. The role of activated macrophages in recovery from and protection against viral infections has not been much studied. Macrophages can produce interferon as mentioned earlier. It has been shown that the high susceptibility of newborn mice to infection with Herpes

simplex virus is correlated with the inability of their macrophages to become activated (Hirsch et al., 1970). Similarly mouse hepatitis virus multiplies inside macrophages from thymectomized mice or certain strains of mice that are congenitally susceptible to infection with this virus. Macrophages from normal resistant mice are capable of limiting the growth of the virus (Allison, 1974). The studies of Blanden (1971a & b) showed that immunity to mousepox could be passively transferred by T-lymphocytes and that macrophages accumulated at virus-infected foci and destroyed the virus in mice treated in this way. Morahan et al. (1977) found that activated but not stimulated mouse macrophages could depress the production of Vaccinia and encephalomyocarditis virus in mouse embryo fibroblast cultures. This inhibition was species-specific and might therefore have been mediated by interferons. These experiments did not differentiate between a direct effect on the virion and an effect on the virus-infected cells. Macrophages have been shown to kill virus-infected cells. Human monocytes exert an antibody-dependent cytotoxic effect on cells infected with Herpes simplex virus (Kohl et al., 1977). In other cases cytotoxicity occurred in the absence of antibody. Thus calf alveolar macrophages are cytotoxic against calf kidney cells infected with parainfluenza 3 virus (Probert et al., 1977). Starch-induced mouse macrophages killed transformed and non-

transformed mouse embryo fibroblasts infected with mouse sarcoma virus, mouse leukemia virus, Sendai or influenza virus (Goldman & Hogg, 1977). The cytotoxic effect was thus not limited to cells that have undergone malignant transformation. Interestingly there was no requirement for H-2 compatibility, implying that macrophages recognize viral antigens alone in contrast to cytotoxic T-cells (Doherty *et al.*, 1976). Rodda & White (1976) showed that infection of mice with a Togavirus resulted in rapid, non-specific activation of macrophages that became cytotoxic for cells infected with various Togaviruses. This response preceded the appearance of immune T-cells and specific antiviral antibody.

There is thus some evidence that macrophages, particularly if activated, may play a role in the defence against some viral infections either by inhibiting the multiplication of viruses inside macrophages themselves or in other cells or by a cytotoxic effect on virus-infected cells.

12.5.3. Cytotoxicity. Cytotoxicity is a major attribute of activated macrophages along with the enhanced antimicrobial activity. This cytotoxic effect can be tested in microcytotoxicity assays in vitro by incubating the macrophages with the target cells. At the end of the incubation period the number of target cells remaining can be counted microscopically. Alternatively the target cells can be prelabelled with radioactive material such as ^{51}Cr and the release measured as an indicator

of cytolysis or the rate of incorporation of tritiated thymidine can be measured as an assay of a cytostatic effect (see McBride, 1978). Macrophages have been shown to kill or inhibit the growth of syngeneic, allogeneic or xenogeneic cells in a non-specific way (Keller, 1976; Evans & Alexander, 1976; Hibbs, 1976). Different cell types vary in their susceptibility to macrophage cytotoxicity, cells that have undergone malignant transformation are most easily affected (Keller, 1976; Hibbs, 1976). Normal cells that are rapidly dividing can, however, also be shown to be killed by activated macrophages (Jones et al., 1975; Holterman et al., 1975) although the more common effect is growth inhibition (Keller, 1976). Susceptibility to macrophage cytotoxicity is correlated with the rate of division (Holterman et al., 1975) and loss of contact inhibition with increased agglutinability by lectins (Hibbs, 1973). It appears therefore that activated macrophages are in these situations reacting to surface characteristics shared by normal mitotic cells and malignant cells (see Gahmberg, 1977). It has, however, recently been shown that normal mouse macrophages can kill and inhibit the growth of chicken embryonic fibroblasts (Cabilly & Gallily, 1977). This is consistent with earlier observations that macrophages can recognize xenogeneic cells as described in a previous chapter. The killing by macrophages of non-malignant cells infected with virus was also mentioned above.

The cytotoxic activity of macrophages is usually non-specific. They can, however, be involved in specific cytotoxic reactions as "armed" macrophages (Evans & Alexander, 1976) or as effector cells in antibody-dependent cytotoxicity (Evans, 1975; Haskill & Fett, 1976).

Macrophage-mediated cytotoxicity is a slower process than killing by cytotoxic T-cells (Lohmann-Matthes, 1976) and the kinetics vary somewhat with different experimental systems. Keller (1974) found that the cytostatic effect of rat macrophages was irreversible within 6-18 hours but that further 6-18 hours were needed to complete the reaction. Killing of tumour cells by rat macrophages took somewhat longer and was detectable only after 12-24 hours (Keller, 1976). In contrast cytolytic macrophages from mice immunized with living allogeneic lymphoma cells cause irreversible damage within 1 hour of contact (Den Otter *et al.*, 1972) whilst the specific cytostatic effect of macrophages from mice immunized with irradiated lymphoma cells took 24 hours to become irreversible (Evans & Alexander, 1976).

12.5.4. Mechanisms of macrophage-mediated cytotoxicity.

Macrophages can be seen to collect around their targets and adhere to them (Hibbs, 1973; Keller, 1976). Cytotoxicity is exerted most effectively when close cell contact is established but phagocytosis of living cells does not seem to be involved (Hibbs, 1973; Keller, 1976; Evans & Alexander, 1976). Several workers have, however, obtained soluble factors that

were either cytostatic or cytolytic from macrophage cultures (Keller, 1976; Reed & Lucas, 1975; Kramer & Granger, 1972). It is not known whether different factors are required for cytostatic and cytolytic effects or whether experimental conditions and the state of target cells determine the final outcome of a reaction with one type of cytotoxic factor. Most of the reported factors are non-specific in their action. McIvor and coworkers have, however, obtained a heat-labile antigen-specific factor that is released by macrophages from mice immunized with allogeneic tumour cells after 2 hours of incubation with the specific target cells in vitro (McIvor & Weiser, 1971; Piper & McIvor, 1975). After longer culture (48 hours) such cultures yield a non-specific cytotoxic factor (Kramer & Granger, 1972). It seems likely that the early specific factor contains a lymphocyte-derived component such as SMAF or cytophilic antibody to render it specific.

Some suggestions have been made about the mechanisms of macrophage-mediated cytotoxicity and the nature of macrophage-derived cytotoxic factors. Hibbs (1974) observed the transfer of lysosomal material from macrophages into attached tumour cells and proposed that lysosomal enzymes might mediate the cytotoxic effect. Ferluga et al. (1976 & 1978) have recently put forward the hypothesis that activated macrophages exert their cytolytic effect by producing C3a. Another recent suggestion is that arginase released by activated macrophages

is responsible for the cytotoxic effect (Currie, 1978). Arginase was shown to be released by mouse macrophages activated in vitro by zymosan or bacterial lipopolysaccharide but not normal or stimulated macrophages.

The question arises whether cytotoxic factors are synthesised constantly by normal macrophages and activation only results in accelerated production and secretion or whether the synthesis of new factor(s) is induced. The expression of cytotoxicity is closely linked in time to accelerated protein synthesis in activated rat macrophages (Keller et al., 1974). The ability of normal macrophages to kill xenogeneic fibroblasts (Cabilly & Gallily, 1977) indicates that the killing mechanisms are already operating at least to some degree in unstimulated macrophages. On activation the synthesis of cytotoxic factors might be stimulated and secretion induced in a manner similar to the effect of activation on the production of lysosomal enzymes and LAF (see above). Surface properties that encourage cell recognition and adherence are probably very important for the action of labile factors and are likely to account for the high susceptibility of malignant cells to macrophage cytotoxicity. It has also recently been shown that activated macrophages bind more avidly to tumour cells in vitro as compared with normal macrophages (Piessens, 1978). The expression of a new surface antigen by cytotoxic macrophages might be relevant in this context (Kaplan et al., 1978).

12.5.5. Significance of macrophage-mediated cytotoxicity.

The role of macrophages in the defence against neoplasia is still very uncertain. Malignant tumours are commonly infiltrated by large numbers of macrophages (Evans, 1977). Treatment of tumour-bearing animals with macrophage-activating agents such as C. parvum, BCG or pyran copolymer may result in inhibition of tumour growth or tumour regression (Scott, 1974a & b; Snodgrass & Hanna, 1973; Morahan & Kaplan, 1976). The route and method of administration of the activating agents appears to be important in these experiments and they are most effective when injected into the tumour site (Scott, 1974a & b; Hibbs, 1976). Macrophages that are cytotoxic in vitro have been shown to prolong the survival of mice when injected locally into a tumour bearing site (Den Otter et al., 1977). Similarly when macrophages activated in vivo with C. parvum are transferred along with tumour cells subcutaneously into sublethally, irradiated syngeneic recipients the development of tumour nodules is delayed and final tumour incidence decreased (McBride et al., 1977). In tumours from animals treated with BCG macrophages can be seen to collect around tumour cells and the tumour cells appear to degenerate following contact with the macrophages (Snodgrass & Hanna, 1973). A high degree of macrophage infiltration in breast cancer has been correlated with a relatively low incidence of metastases (Lauder, 1977). Using an experimental model of metastasation

where tumour cells are injected intravenously into mice and the number of tumour nodules formed in the lungs is counted. Milas et al. (1975) showed that pretreatment of the mice with C. parvum reduced the number of nodules formed and prolonged the survival of the animals.

Many tumours or their products can depress macrophage function (see James, 1977). It is possible that whilst macrophages may not be able to attack a large tumour load effectively they may play some role in the inhibition of development of malignant lesions and prevention of metastatic spread of cancer (see Evans, 1977).

13. MECHANISMS OF MACROPHAGE ACTIVATION

This chapter reviews briefly the processes that are thought to mediate signal transmission across the plasma membrane when cells respond to external stimuli and discusses which mechanisms are likely to be involved in macrophage activation.

13.1 Mechanisms of cellular stimulation.

13.1.1. Signal-response coupling. The functions of living cells can be influenced by external stimuli. This often takes the form of a chemical reaction of an influencing substance such as a hormone with a receptor on the cell surface although specialized sensory nerve cells are able to respond to physical stimuli such as pressure, temperature changes or sound. The initial reaction gives rise to a series of events that transmit the signal to the interior of the cell and results in the final triggering of a specific response such as an action potential, contraction, synthesis, secretion etc. This is known as signal-response coupling (see Mountcastle, 1974).

13.1.2. Ion fluxes and electrophysiological phenomena. In the case of nerve cells an electrical pulse or interaction with neurotransmitter substances gives rise to the electrical response of an action potential based on rapid changes in the membrane permeability to Na^+ and K^+ ions and this in turn triggers the release of transmitter substances at

the efferent end. Specialized sensory cells may respond directly to physical stimuli such as pressure by a change in ion permeability that results in the electrical potential changes (see Mountcastle, 1974). Acetylcholine released from nerve endings at the motor end plate of striated muscle initiates changes in permeability to Na^+ and K^+ ions in the plasma membrane of the muscle cell thus creating an action potential. This signal is translated into the contractile response by several steps. The action potential causes the release of Ca^{++} ions from intracellular stores in the sarcoplasmic reticulum. The Ca^{++} ions then bind to troponin and release its depressive effect on the contractile filaments. This finally results in the activation of the actomyosin ATP-ase and parallel sliding movement of the actin and myosin filaments (Fuchs, 1974; Ebashi, 1976). Noradrenalin released from sympathetic nerve fibres can have twofold effects depending on the nature of the tissue receptors. Interaction with α -adrenergic receptors probably leads to an increase in permeability to Na^+ ions with production of an action potential in smooth muscle in a manner similar to the effect of acetylcholine on striated muscle. The β -adrenergic receptors on smooth muscle mediate an increase in permeability to K^+ ions with stabilization of the membrane potential as well as an increase in cellular levels of cyclic AMP (see Goodman & Gilman, 1975). The rapid electrophysiological phenomena described above are associated with the propagation

of nervous stimulation and motility responses but not longer term metabolic stimulation.

13.1.3. Mode of action of steroid hormones.

Steroid hormones appear to enter cells either by diffusion through the lipid bilayer of the plasma membrane or by a protein-mediated transport mechanism. They combine with a specific receptor molecule in the cytoplasm and this hormone-receptor complex then seems to be transferred to the nucleus where it initiates the synthesis of specific proteins (Gorski & Gannon, 1976).

13.1.4. Cyclic nucleotides as second messengers.

Other hormones stay on the cell surface and their action is often mediated by a second messenger of which cyclic adenosine monophosphate (cyclic AMP) was the first to be discovered and is best studied. Cyclic AMP mediates the β -adrenergic metabolic effects of catecholamines and the action of several peptide hormones such as glucagon, ACTH, LH and TSH (Robison *et al.*, 1968; Cuatrecasas, 1974; Catt & Dufau, 1977). These hormones can be shown to cause an increase in the intracellular levels of cyclic AMP and their action can be mimicked by cyclic AMP. The hormone receptor may in some cases be a permanent part of the enzyme adenyl cyclase which catalyses the formation of cyclic AMP from ATP, in other cases the receptor combines

with the catalytic subunit of the enzyme after binding the hormone. The latter process is dependent on the fluidity of membrane lipids and may explain how several different hormones can stimulate adenyl cyclase even on the same cell (Cuatrecasas, 1974; Rimon et al., 1978). The activation of the adenyl cyclase which also requires the presence of guanyl nucleotides leads to increased intracellular levels of cyclic AMP which in turn stimulates intracellular protein kinases and results in a cascade of enzyme activations (Robison et al., 1968; Helmreich et al., 1976; Catt & Dufau, 1977).

Another cyclic nucleotide, cyclic guanosine monophosphate (cyclic GMP), has been implicated in signal response coupling. The action of cyclic GMP is not as straightforward as that of cyclic AMP, intermediate steps are less well understood and it may be involved by mechanisms more complex than simple mass action. In contrast to the direct stimulation of adenyl cyclase by hormones guanyl cyclase can not be directly stimulated by agents that raise the intracellular levels of cyclic GMP (Goldberg & Haddox, 1977). An increase in the intracellular levels of cyclic GMP may mediate some of the metabolic effects of muscarinic cholinergic as well as α -adrenergic stimulation. The effects of cyclic AMP and cyclic GMP in one tissue frequently oppose each other, one being inhibitory and the other stimulatory. This has been called a yin-yang relationship (Goldberg & Haddox, 1977). Examples include the stimulatory

effect of cyclic GMP on lymphocyte proliferation opposed by the inhibitory effect of cyclic AMP (Watson et al., 1973; Watson, 1975) and the opposing effects on cardiac contractility (Nawrath, 1976). It has been suggested that the ratio of the intracellular concentrations of cyclic AMP and cyclic GMP is more important than the absolute level of one or the other in determining stimulation or inhibition of cellular processes (Watson et al., 1973; Watson, 1975).

13.1.5. Role of calcium ions in signal transmission.

Calcium ions play an important role in cellular signal transmission and changes in the intracellular concentration and distribution of Ca^{++} are closely linked to the action of cyclic nucleotides. Calcium is required for the action of some enzymes that are stimulated by increased cyclic AMP levels (Rasmussen, 1970). Cyclic AMP can stimulate both the influx and efflux of Ca^{++} and redistribution of Ca^{++} within the cells (Rasmussen, 1970; Berridge, 1975) and Ca^{++} may have a feedback regulating effect on the level of cyclic AMP by stimulating phosphodiesterase which breaks down cyclic AMP or inhibiting the activity of adenyl cyclase (Berridge, 1975; Helmreich et al., 1976). The increase in cyclic GMP levels caused by acetylcholine is dependent on Ca^{++} and a Ca^{++} ionophore can itself raise the concentration of cyclic GMP in exocrine pancreatic cells (Christophe et al., 1975). Calcium ions also influence the

permeability of plasma membranes to other ions and affect the activity of many enzymes (Rasmussen, 1970; Williams, 1970; Berridge, 1975).

13.1.6. Turnover of phosphatidylinositol.

Several years ago Hokin & Hokin (1958) found that stimulation of exocrine pancreatic cells caused a marked increase in the uptake of inorganic phosphorus into the membrane phospholipid phosphatidylinositol whilst far less uptake occurred into other and more abundant membrane phospholipids. This observation was subsequently extended to many other tissues and it appeared to be an early event in cellular stimulation without being linked to the cyclic AMP pathway (see Michell, 1975). It now seems clear that the increased labelling of membrane phosphatidylinositol reflects an accelerated turnover of the phosphorylinositol headgroup of phosphatidylinositol and that many hormones and pharmacological agents stimulate the breakdown of phosphatidylinositol into diglyceride and phosphorylinositol. The enzymes concerned with the turnover of the phosphorylinositol headgroup are partly membrane bound and appear to be enriched in the plasma membrane whilst the enzymes involved in the de novo synthesis of phosphatidylinositol are found in the endoplasmic reticulum (Michell, 1975). Turnover is stimulated by contact with a surface receptor as stimulation can be inhibited by an appropriate antagonist (Hokin & Sherwin, 1957; Hokin, 1968; Michell, 1975) although there is no firm evidence that binding

to a receptor leads directly to the activation of enzymes involved in the turnover. The enhanced turnover can often be observed within a few minutes of applying the stimulus and precedes other metabolic changes brought about by the stimulant (Hokin, 1968; Fisher & Mueller, 1968; Michell, 1975). All these observations indicate that increased turnover of phosphatidylinositol may play an important role in the transmission of signals across the plasma membrane. The model of phosphatidylinositol turnover shown in Figure 1 is taken slightly modified from the review by Michell, 1975. It includes specific phospholipid exchange proteins that transfer phospholipids between the plasma membrane and their site of resynthesis, the endoplasmic reticulum.

As the external stimulus probably acts by inducing the cleavage of phosphatidylinositol into phosphorylinositol and diglyceride the most direct way of measuring the effect is to prelabel the cells' phosphatidylinositol by incubation with radioactive phosphorus and measure the rate of breakdown (see Michell, 1975). It is, however, more common to measure the rate of uptake of radioactive phosphorus or myo-inositol into phosphatidylinositol which reflects the rate of turnover as only the diglyceride backbone appears to be directly reutilized in the cycle (Hokin, 1968; Fisher & Mueller, 1968 & 1971; Michell, 1975; Shearer & Crouch, 1977).

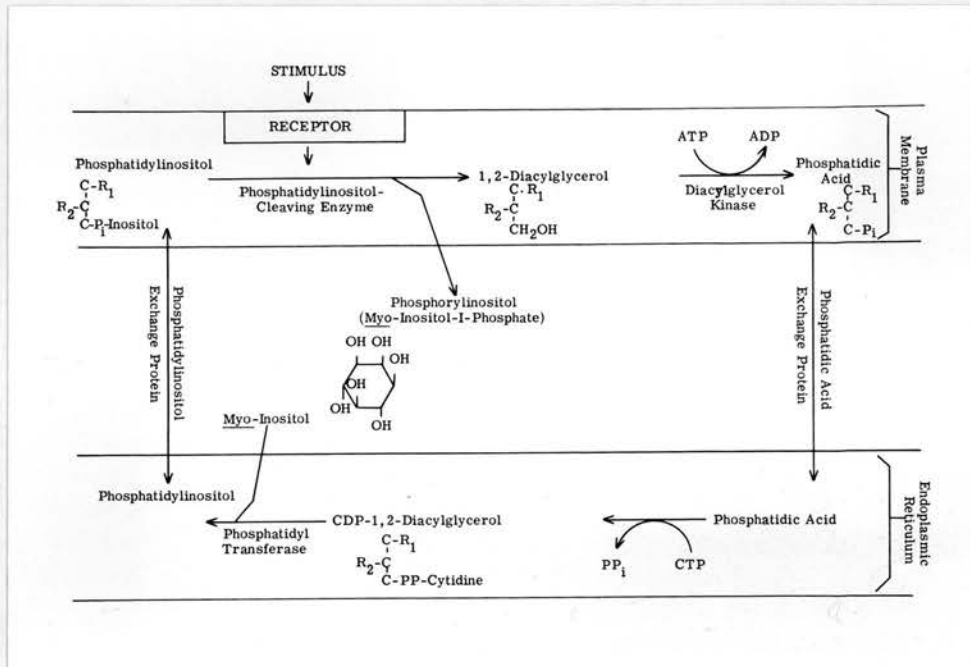


Figure 1. Model of cellular turnover of phosphatidylinositol indicating enhancement of the activity of phosphatidylinositol cleaving enzyme in response to an external stimulus. (Derived from Michell, 1975).

Lapetina & Michell (1973) have suggested that the phosphorylinositol headgroup that has been cleaved off phosphatidylinositol might give rise to the formation of the derivative inositol-1,2,-cyclic phosphate that might act as a second messenger but there is little experimental evidence for such a mechanism.

The stimulation of turnover of cellular phosphatidylinositol is not affected by either Ca^{++} -chelating agents or Ca^{++} -ionophores

(Hokin & Hokin, 1965; Jones & Michell, 1975) but isolated soluble phosphatidylinositol-cleaving enzymes from lymphocytes require a very low concentration of Ca^{++} ions (Allan & Michell, 1974a & b). The amount of enzyme present in lymphocytes is far in excess of that required for the observed increase in phosphatidylinositol turnover following stimulation. Intracellular Ca^{++} ions would be sufficient to support this activity and it is unlikely that the rate of phosphatidylinositol turnover could be controlled by fluxes in Ca^{++} -concentration (Allan & Michell, 1974b; Michell, 1975). Many of the stimulation processes where an increased turnover of phosphatidylinositol occurs are dependent on extracellular Ca^{++} and this reaction would thus have to take place either before or alongside the processes involving Ca^{++} ions (Michell, 1975). The phosphorylated derivatives of phosphatidylinositol, diphospho- and triphospho-inositides, that are found in plasma membranes show an affinity for Ca^{++} and other divalent cations and it has been suggested that these derivatives might play a role in regulating the fluxes and distribution of Ca^{++} ions (Hawthorne, 1972). Increased turnover of phosphatidylinositol is clearly linked to stimuli that cause increased levels of intracellular cyclic GMP but leave the concentration of cyclic AMP unchanged or decreased. Michell *et al.* (1977) have recently put forward the hypothesis that interaction with a surface receptor such as the muscarinic cholinergic receptor causes an accelerated

turnover of phosphatidylinositol which then leads to the opening up of Ca^{++} -gates resulting in an increase in available Ca^{++} intracellularly stimulating the production of cyclic GMP.

As many different cell types utilize the same mechanisms for signal transmission the specificity of the response must be determined at the level of the surface receptor and/or specialized metabolism of differentiated cells (Rasmussen, 1970; Cuatrecasas, 1974; Catt & Dufau, 1977).

13.2 Mechanisms of macrophage activation.

13.2.1. Cyclic nucleotides as mediators of macrophage activation. Macrophages have not been much studied with respect to their early responses to activating stimuli. Remold-O'Donnell & Remold (1974) demonstrated that lymphokines stimulated the activity of membrane adenylyl cyclase in guinea pig macrophages after 24 hours of culture. It was, however, shown later that this is not accompanied by an increase in the intracellular concentration of cyclic AMP. Higgins et al. (1976) found that the level of cyclic AMP in guinea pig macrophages remained unchanged when measured at 3 minutes, 1 hour and 24 hours after adding the MIF-rich fraction of a lymphocyte supernatant. Pick (1977) detected a decrease in the cyclic AMP content of guinea pig macrophages following exposure to lymphokines, this was most marked after 1-2 hours of incubation. The action of lymphokines on macrophages is thus not likely to be mediated by

an increase in intracellular cyclic AMP. In addition Koopman et al. (1973) found that exogenous cyclic AMP, its analogues or agents that raise intracellular cyclic AMP levels prevent the migration inhibition response of guinea pig macrophages to lymphokines. Exogenous cyclic AMP or exposure to agents that lead to an increase in cyclic AMP levels have also been shown to depress other macrophage functions. Thus the release of lysosomal enzymes following phagocytosis of zymosan is inhibited and a higher concentration of cyclic AMP also depresses phagocytosis (Koopman et al., 1973; Welscher & Cruchaud, 1976). Production of plasminogen activator by thioglycollate-stimulated macrophages is also inhibited (Vassalli et al., 1976) as well as the increase in the number of Fc-receptors following stimulation in vitro with heterologous serum (Rhodes, 1975b). Agents that act through the cyclic AMP pathway may thus have a regulating effect on macrophage function.

Cyclic GMP appears to be a more likely candidate for a second messenger of macrophage activation although there is no firm evidence. Diamantstein & Ulmer (1975 & 1976) found that exogenous cyclic GMP stimulated the release of LAF by mouse macrophages. Cyclic GMP or agents that raise the intracellular levels of cyclic GMP had, however, no effect on the production of plasminogen activator by stimulated macrophages (Vassalli et al., 1976) perhaps only indicating that the production was

already maximally stimulated. Human transfer factor, serotonin, carbamylcholine and ascorbic acid have been shown to cause an increase in the intracellular concentration of cyclic GMP in human blood monocytes (Sandler et al., 1975a, b & c) and all these substances apart from transfer factor were shown also to enhance the chemotactic response of monocytes to bacterial endotoxin (Sandler et al., 1975c).

It is interesting to note here that cyclic AMP has an inhibitory effect on several aspects of immune and inflammatory responses (Bourne et al., 1974) whilst cyclic GMP tends to be stimulatory. Thus lymphocyte proliferation and antibody production is stimulated by cyclic GMP and depressed by cyclic AMP (Watson et al., 1973; Watson, 1975) and T-cell cytotoxicity is inhibited by raised levels of cyclic AMP (Henney & Lichtenstein, 1971). Release of lysosomal enzymes from neutrophils is inhibited by an increase in cyclic AMP (Zurier et al., 1973; Ignarro et al., 1974) and their ability to kill Candida is depressed by cyclic AMP (Bourne et al., 1971). The response of polymorphonuclear leucocytes to a chemotactic stimulus is enhanced by cyclic GMP (Estensen et al., 1973).

13.2.2. Role of divalent cations in macrophage activation. Hand et al. (1977) and Gordon et al. (1977) have recently described the effects of treatment with the divalent

cation ionophore A23187 on macrophage function. Hand et al. (1977) used normal rabbit alveolar macrophages and found that exposure to the ionophore induced changes characteristic of macrophage activation including enhanced capacity to kill Listeria monocytogenes. Somewhat unexpectedly the response was more sensitive to a depletion of Mg^{++} ions than Ca^{++} ions. In the experiments of Gordon et al. (1977) A23187 was found to be toxic for thioglycollate-induced mouse macrophages. Zymosan-induced release of lysosomal enzymes occurred in a medium devoid of Ca^{++} or Mg^{++} ions and was stimulated by 0.3 - 5 mM Mg^{++} but inhibited by the same concentration of Ca^{++} thus again placing more emphasis on the role of Mg^{++} as compared with Ca^{++} . These results seem to give rise to more questions than they answer and it is for example possible that an intracellular pool of Ca^{++} may be found to be more important in macrophages than extracellular Ca^{++} . Romeo et al. (1975) have studied the role of ion fluxes in the stimulation of metabolism in neutrophils and found that Ca^{++} -ionophores and particularly an ionophore that transports both Ca^{++} and K^{+} ions stimulated O_2 -utilization and glucose metabolism in a manner similar to a phagocytic stimulus.

13.2.3. Changes in phospholipid metabolism during macrophage activation. Karnovsky and coworkers have studied the effects of phagocytosis on the incorporation of radioactive

inorganic phosphorus into cellular phospholipids. Most of their studies were performed with neutrophils. They found that phagocytosis of starch particles and exposure to the surface active agents endotoxin, deoxycholate and digitonin all caused an increased uptake of inorganic phosphorus into phosphatidic acid and more markedly into a phospholipid fraction containing phosphatidylserine and phosphatidylinositol (Karnovsky et al., 1961; Graham et al., 1967). Casein-induced guinea pig macrophages responded in a similar way to these stimuli. Sastry & Hokin (1965) also found that phagocytosis of starch and latex stimulated the uptake of radioactive inorganic phosphorus into phosphatidic acid and phosphatidylinositol and inositol into phosphatidylinositol in neutrophils whilst incorporation of glycerol or fatty acids and de novo synthesis of phosphatidic acid were unchanged. These results may indicate that enhanced turnover of phosphatidylinositol plays a role in the stimulation of phagocytic cells including macrophages and this would be in accordance with a stimulatory function of cyclic GMP in these cells.

13.2.4. Rapid ion fluxes. It seems possible that the rapid responses of phagocytes involving cellular motility, viz. chemotaxis and phagocytosis, are initiated by rapid ion fluxes and electrophysiological changes reminiscent of the stimulation of muscle contraction. This has already been discussed in an earlier chapter (9).

14. OUTLINE OF THE PRESENT STUDY OF RECOGNITION BY MACROPHAGES AND THEIR ACTIVATION

The original aim of this study was to investigate the early events following exposure of macrophages to activating agents. It was reasoned that the activating agent would first have to make contact with the macrophage surface and that therefore some type of recognition mechanism was involved. The agent initially chosen for the study was Corynebacterium parvum. In preliminary experiments direct binding of this micro-organism to the surface of normal mouse peritoneal macrophages was observed. Bacterial cell walls are largely composed of carbohydrates and it was also known that an antigenic determinant in the cell wall of C. parvum was mainly carbohydrate. The composition of this antigen had been analysed (Dawes et al., 1974). Recognition of carbohydrates on the bacterial surface was therefore considered a likely mechanism leading to binding especially in view of some recent evidence for the involvement of carbohydrates in cellular adhesion phenomena. This hypothesis was tested in a series of inhibition experiments using different sugars and the results of these experiments appeared to confirm the original hypothesis. It was realised that this type of recognition would probably be non-specific and represent a mechanism for the recognition of a wide range of non-opsonized bacteria by phagocytes. To test this idea

the same type of inhibition experiment was performed using several different bacteria, including some strains with a well defined cell wall composition. A study of such a recognition mechanism was thought to be relevant to the understanding of the general ability of phagocytes, even of primitive species, to distinguish non-self from self. The requirement of the binding of C. parvum for divalent cations was investigated. An attempt was made to analyse the nature of the plasma membrane molecules of the macrophages involved in the binding of non-opsonized bacteria by testing the effect on the binding of C. parvum of exposure of the macrophage surface to various enzymes and chemicals.

The second part of this study dealt with the consequences of binding of activating agents to the macrophage surface. A possible mechanism for transmission of an activating signal across the macrophage plasma membrane was explored. It was decided to examine the influence of activating stimuli on the turnover of phosphatidylinositol. This had been shown to be increased in connection with signal transmission in a number of different cell types including lymphocytes and there were also indications that it was enhanced in stimulated phagocytes. The uptake of radiolabelled myo-inositol over a defined period was measured as an estimate of the rate of turnover of phosphatidylinositol. The influence of two activating agents,

C. parvum and bacterial lipopolysaccharide was studied and compared with the effect of several inert particles. In an attempt to establish further the link with activation the effect of treatment of macrophages with bacterial lipopolysaccharide on their ability to inhibit the growth of Listeria monocytogenes was also tested. The effect of lipopolysaccharide and various test particles on the synthesis and secretion of three lysosomal enzymes was also investigated.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. ANIMALS

In the first sets of experiments (studying the binding of C. parvum to macrophages, the inhibition of binding by sugars and the effect of divalent cations) cells from CBA mice were used. These mice were obtained from the Centre for Laboratory Animals, Edinburgh University, The Bush, Nr. Penicuik, Midlothian. These mice were 5-10 weeks old and specific pathogen free (SPF). For all subsequent experiments cells from mice of the C3H/Buf strain (also SPF) age 6-12 weeks were used. These animals were from the departmental breeding colony and are held under conventional conditions. In each experiment animals of the same strain, sex and age were used. It was found that the yield of peritoneal exudate cells was usually somewhat higher from females than from males and, therefore, females were preferably used.

Twice during this study the mice became infested with nematodes (Aspiculuris tetratera) which had a stimulatory effect on their peritoneal exudate macrophages. This was in each case successfully treated with Piperazine with a return of the macrophages to normal. The use of macrophages was avoided until the mice had been cleared of the nematodes. Towards the end of this study the mice were periodically affected by

an unidentified infection manifested as decreased weight gain in young mice and conjunctivitis. These animals were also often found to have enlarged spleens. Routine bacteriological examination yielded no conclusive results. This infection caused some difficulties in obtaining normal resting macrophages for some of the last experiments of this study as will be mentioned later.

2. BACTERIA AND OTHER PARTICLES

2.1 Bacteria.

Corynebacterium parvum (strain 10390) and Propionibacterium freudenreichii (strain 10470) were originally obtained from the National Collection of Type Cultures (NCTC), Colindale Avenue, London and maintained in the laboratory by subculturing anaerobically on horse blood agar. For use in the experiments these two organisms were grown anaerobically in horse digest broth, containing 3% glucose (see Dawes et al., 1974). Staphylococcus albus, Staphylococcus aureus, Streptococcus viridans, Streptococcus pyogenes, Streptococcus pneumoniae, Bacillus anthracoides, Listeria monocytogenes and Escherichia coli were all obtained from the departmental teaching collection. Pseudomonas aeruginosa, NCTC 578 (a mucoid and a non-mucoid strain) were a kind gift from Dr. John Govan in the Department of Bacteriology. Klebsiella aerogenes, (wild type, serotype 2, NCTC 5055), a non-capsulated mutant (M2) and a rough mutant (M10B)

as well as the following strains of Salmonella typhimurium: SL 1542, with a complete lipopolysaccharide; three rough mutants with incomplete lipopolysaccharides, SL 1096 (RfaK, chemotype Rb), SL 1099 (chemotype Rc) and SL 1102 (RfaE, chemotype Re) were kindly provided by Dr. Ian Sutherland, Department of Microbiology, University of Edinburgh. All these bacteria were grown aerobically in nutrient broth except for the Streptococci which were grown in horse digest broth. All bacteriological media were obtained from the departmental media preparation rooms.

To obtain bacterial suspensions for use in the experiments the bacteria were grown for two days in 10-15 ml of the appropriate medium and then transferred into 100 ml of the same medium and culture continued overnight. The cultures were then centrifuged for 15-20 minutes at 2500g at 4°C, washed once in physiological saline and killed by 24 hours exposure to 0.5% formalin in saline (100 ml) at 4°C. The killed organisms were washed 4 times in saline and finally resuspended in 2-4 ml of saline. These stock suspensions were kept without preservatives and could be stored at 4°C for up to 3-4 weeks. As Listeria monocytogenes was not used in the binding assay but only to test the antimicrobial activity of macrophages this organism was used live; after overnight culture in 10-15 ml of nutrient broth the organisms were washed three times in Dulbecco's phosphate buffered saline before use. Control experiments were also performed to test the binding of living bacteria to

macrophages the cultures being prepared in the same way as described for L. monocytogenes.

Bacteria were counted in a Neubauer chamber (improved), 0.01 mm depth. The concentration of C. parvum was, however, estimated photometrically, as it had been shown that the optical density reading of 0.38 at 540 nm corresponded to 3×10^9 C. parvum per ml.

2.2 Other particles.

Polystyrene latex particles (0.81 μ m diameter) were obtained from Difco Laboratories, Detroit, Michigan. A stock suspension of colloidal carbon was a kind gift from the Department of Pathology, University of Edinburgh.

The latex particles were counted microscopically in the same way as described for bacteria. The colloidal carbon was diluted one in ten for use to a final concentration giving the optical density reading of 0.36 at 540 nm.

3. REAGENTS

3.1 Tissue culture medium.

Tissue culture medium 199 or Eagle's minimal essential medium (Eagle's MEM) both from the Wellcome Research Laboratories, Beckenham, Kent were used. These culture media were buffered with 1 ml 2.5 M HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid, Calbiochem, La Jolla, California) and 2 ml 4.4% sodium bicarbonate per 100 ml and the pH adjusted by eye to a

light pink colour with a few drops of 2 N NaOH. For short term cultures (less than 4 hours at 37°C) no antibiotics were included but for longer culture periods penicillin and streptomycin were added to a final concentration of $50\text{ }\mu\text{g/ml}$ of each.

Foetal calf serum was obtained from Sera-Lab, Crawley Down, Sussex. The serum was heat-inactivated for 30 minutes at 56°C before use.

For the harvesting and washing of cells as well as the assay for binding of bacteria to macrophages Dulbecco's phosphate buffered saline (Dulbecco's PBS) was used, often supplemented with solution B, containing Ca^{++} - and Mg^{++} -ions (Dulbecco's PBS + B). Dulbecco's PBS contains 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 , 0.2 g KH_2PO_4 and 2 drops 1% aqueous phenol red per litre and Dulbecco's PBS + B contains a final concentration of 10^{-3} M of Ca^{++} and $0.5 \times 10^{-3}\text{ M}$ of Mg^{++} .

3.2 Sugars, sugar derivatives and other components of bacterial cell walls.

The aldohexoses D-glucose, D-galactose and D-mannose; the ketohexose D-fructose; the deoxy-hexoses L-fucose and L-rhamnose; and the pentoses D-arabinose and D-xylose were obtained from British Drug Houses, Poole, Dorset, as well as the sugar alcohols sorbitol, mannitol, inositol and glycerol and the sodium salts

of the carbohydrate metabolites succinate, pyruvate and acetate. N-acetylneuraminic acid (Koch Light Laboratories, Colnbrook, Buckinghamshire) was a kind gift from the Microbial Pathogenicity Research Laboratory, Department of Bacteriology, and the amino sugars glucosamine and galactosamine as well as N-acetylglucosamine and the sugar acid glucuronic acid were kindly provided by Jim Doyle, Department of Biochemistry, University of Edinburgh. 2-Keto-3-deoxyoctonate was obtained from Sigma Chemical Company, St. Louis, Missouri, and Lipid A, prepared from phenol-extracted lipopolysaccharide from K. aerogenes (Poxton & Sutherland, 1976) was kindly provided by Dr. Ian Sutherland, Department of Microbiology, University of Edinburgh.

These substances were made up to 310 mEq solutions in sterile distilled water and then diluted in Dulbecco's PBS + B to a final concentration of 10 mM for use in the binding inhibition experiments. Dried lipid A had to be dissolved in saline with the addition of small amounts of NaOH (0.5 N). The pH was then adjusted to 7.2 with HCl and Ca^{++} - and Mg^{++} -ions added as chlorides to a final concentration of 2 mM of each.

Lipopolysaccharide W (endotoxin) from Escherichia coli 055:B5 was obtained from Difco Laboratories, Detroit, Michigan. A stock solution of 1 mg/ml in sterile distilled water was prepared and diluted to the required concentration in Eagle's MEM for use as activating agent in the assay for uptake of myo-(2- ^3H)-inositol and in tests for antibacterial activity and lysosomal enzyme levels.

3.3 Enzymes.

Enzymes were obtained from the following sources: Pronase (Streptomyces griseus), β -galactosidase (Saccharomyces fragilis) and neuraminidase (Vibrio cholerae) were obtained from the British Drug Houses, Poole, Dorset; phospholipase A (Vipera russeli), phospholipase C (Clostridium perfringens) and phospholipase D (cabbage) from Koch Light Laboratories, Colnbrook, Buckinghamshire; and trypsin from Difco Laboratories, Detroit, Michigan. These enzymes were used without further purification.

Trypsin, pronase, neuraminidase and phospholipase C were dissolved and diluted in Dulbecco's PBS + B, pH 7.2, β -galactosidase was dissolved and diluted in Dulbecco's PBS + B, containing in addition 0.05 mM Mn^{++} , pH 7.2. Phospholipase A was stored in 50% glycerol and diluted for use in physiological saline containing 10 mM Ca^{++} , pH 7.4, and phospholipase D was dissolved and diluted in Dulbecco's PBS + B adjusted to pH 5.6.

3.4 Radioactive chemicals.

Myo-(2- 3H)-inositol and (6- 3H)-thymidine were obtained from the Radiochemical Centre, Amersham, Buckinghamshire. The specific activity of myo-(2- 3H)-inositol was 2.8 Ci/mmol and 5.0 Ci/mmol in two different batches, and its chemical purity was 98%. Myo-(2- 3H)-inositol was diluted one in ten in Dulbecco's PBS for storage at 4°C as a stock solution and then

diluted to the required concentration in the appropriate tissue culture medium for use. Tritiated thymidine (specific activity approximately 2 Ci/mmol) was diluted directly in tissue culture medium.

3.5 Other chemicals and buffers.

Ethylenediaminetetraacetate (EDTA) as disodium salt was made up to 80 mM in distilled water and then diluted to 10 mM in Dulbecco's PBS or added to tissue culture medium (199 or Eagle's MEM) to yield a final concentration of 10 mM. Sodium periodate (NaIO_4) and potassium borohydride (KBH_4) were dissolved in distilled water to give 150 mM solutions and then diluted in Dulbecco's PBS for use.

For chemical fixation of macrophage monolayers a 0.25% solution of glutaraldehyde in water was used and applied for 9 minutes at room temperature.

Cytochalasin B was obtained from Calbiochem, San Diego, California, and used at a concentration of 5 $\mu\text{g}/\text{ml}$ in Dulbecco's PBS + B or tissue culture medium containing a final concentration of 0.1% dimethylsulphoxide (DMSO) (British Drug Houses, Poole, Dorset) which was used to dissolve the cytochalasin B.

The following buffers were used in antigen-antibody titrations: glycine buffer composed of 0.1 M glycine and 0.1 M NaCl, adjusted to pH 8.2 with 0.1 N NaOH. For use in the latex agglutination test bovine serum albumin (Armour

Pharmaceutical Company, Eastbourne, Sussex) was added to this buffer to a final concentration of 0.1%. Veronal buffer was made by dissolving 31.7g sodium barbitol in 900 ml of distilled water and diluting this again 1 to 4 with distilled water, pH was adjusted to 8.2 with 1 N HCl. For the preparation of gel slides for use in counterimmunoelectroosmophoresis a 1% solution of agarose (Indubiose ^(R), L'Industrie Biologique Francaise, S.A. Gennevilliers, France) was made up in this buffer.

A buffer used for lysing red blood cells was composed of 0.155 M NH_4Cl , 0.1 mM EDTA and 0.01 M KHCO_3 (Ting et al., 1975).

4. PREPARATION OF DERIVATIVES OF CORYNEBACTERIUM PARVUM

4.1 Preparation of culture supernatant.

During culture of C. parvum cell wall antigens are released into the medium (Dawes et al., 1974). In order to obtain high concentrations of this antigen the organisms were allowed to grow into stationary phase before harvesting. The cultures were centrifuged at 2500g for 20 minutes and the supernatant collected. The pH was adjusted to 7.2 before use. This supernatant was usually used directly but sometimes it was dialyzed overnight at 4°C against physiological saline.

4.2 Preparation of acid and lipid extracts.

An acid extract of C. parvum was prepared by incubating equal volumes of the stock suspension of C. parvum and 0.5 N HCl

at 60°C for 1 hour. The organisms were then removed by centrifugation at 2500g at 4°C for 20 minutes, the pH adjusted to 7.2 with 1 N NaOH and the extract dialyzed against physiological saline for 3 hours at 4°C (Dawes et al., 1974; McBride et al., 1976).

A lipid extract of C. parvum was obtained by adding 1 volume of chloroform and 2 volumes of methanol to 0.8 volumes of the stock suspension of C. parvum (see McBride et al., 1976). This mixture was left on ice for 1 hour after which 1 volume of physiological saline was added. After the phases had separated the organic phase was collected from underneath the water-soluble phase and dried under vacuum. The dried extract was then redissolved in 5 ml of physiological saline with the addition of small amounts of 0.5 N NaOH as required, the pH was adjusted to 7.2 with HCl and the extract dialyzed against physiological saline at 4°C for 3 hours.

5. PREPARATION OF ANTI-C. PARVUM ANTISERUM.

Mouse antiserum against C. parvum was raised by injecting 3×10^{10} formalin-killed organisms in 0.1 ml physiological saline i.p. into mice (Woodruff et al., 1974). The animals were killed 10 - 14 days later, bled and the serum collected.

Rabbit-anti-C. parvum antiserum was kindly supplied by Dr. Bill McBride in the Department of Bacteriology. This antiserum

was used in the counterimmunoelectrophoresis and as a positive control in the latex agglutination test. New batches of C. parvum were also tested against this antiserum from time to time to ensure that antigenic properties had been retained during subculture.

6. PREPARATION OF LIPID CARRIER FROM MOUSE LIVERS

The lipids were extracted from 25g of homogenized mouse livers with 50 ml of a 2:1 mixture of chloroform and methanol for 20 minutes at 37°C. After the addition of 25 ml of 0.5 M $MgCl_2$ and thorough mixing the mixture was centrifuged for 10 minutes at 300g. The use of $MgCl_2$ reduces the loss of polar lipids, including phospholipids, into the aqueous phase (Folch et al., 1957). The upper, aqueous, phase was discarded and the organic phase withdrawn from underneath the compressed liver tissue. The organic phase was then equilibrated against 0.5 M $MgCl_2$ which was added without mixing and the phases separated by centrifugation. The aqueous phase was again discarded and residual water removed from the organic phase with anhydrous Na_2SO_4 . The organic phase was finally evaporated to dryness under vacuum and redissolved in 2:1 chloroform-methanol to give a concentration of extract from 2g of liver per ml and stored at -20°C. For use in the assay for uptake of myo-(2-³H)-inositol the extract was diluted one in ten in 2:1 chloroform-methanol.

7. ANTIGEN-ANTIBODY TITRATION TECHNIQUES

7.1 Passive latex agglutination test.

The antibody titre of mouse sera against C. parvum was measured using a latex agglutination technique as described by Woodruff et al. (1974). Latex particles were coated with C. parvum by incubating 1 volume of latex particles with 10 volumes of a stock suspension of C. parvum for 15 minutes at room temperature. This suspension was then diluted one in twenty in glycine buffer, pH 8.2. Doubling dilutions of serum were made in a V-bottom microtitre plate using glycine buffer pH 8.2 containing 0.1% bovine serum albumin as diluent. One drop of diluent and one drop of the particle suspension was then added to each well. The microtitre plate was incubated for 2 hours at 37°C and then kept overnight at 4°C and read the following morning. In order to get a clearer reading the plates were sometimes centrifuged for 5 minutes at 100g and then shaken gently. The titre was recorded as the last well showing clear agglutination.

This test was also used for testing new batches of C. parvum.

7.2 Counterimmunoelectroosmophoresis.

This technique was used to estimate the antigenic activity of the soluble derivatives of C. parvum. The method had been adapted by Dawes et al. (1974) from Prince & Burke (1970). Gel slides were prepared by pouring 10 ml of a melted solution

of 1% agarose in veronal buffer, pH 8.2, on to 8 cm² glass slides that had been precoated with agar by allowing a 1% solution of agar in water to dry out. Holes of 2 mm diameter were punched into the hardened gel by means of suction. The holes were set in rows, 1 cm apart. Doubling dilutions in veronal buffer pH 8.2 were prepared of the samples to be tested and placed into a row of wells in 10 μ l amounts. The same amount of undiluted rabbit anti-C. parvum antiserum was then added to the wells of the next row. The slides were placed in an electrophoresis apparatus containing the veronal buffer and contact established with the slides with wet filter paper. The electrophoresis was run at 250 V for 1-1 $\frac{1}{2}$ hours at 4°C and \leq 40 mA. In this system the antigen travels towards the anode and the antibody towards the cathode. Precipitation bands could be seen immediately after electrophoresis but became clearer after the gels had been kept overnight in a wet chamber at room temperature. The antigen titre was read as the last dilution giving a clear precipitation band with the antiserum.

8. COLLECTION AND CULTURE OF MOUSE PERITONEAL MACROPHAGES

8.1 Collection of mouse peritoneal exudate cells.

Normal mice of the same age and sex were killed by exposure to diethyl ether. The abdominal skin was slit and retracted from the peritoneum and 2.5 ml of Dulbecco's PBS containing 10 i.u./ml of heparin injected i.p. through the midline. After gentle massage for approximately one minute the peritoneal fluid was withdrawn by means of a syringe from the pocket lateral to

the spleen (Stuart et al., 1973). This cell-containing fluid was kept on ice and centrifuged at 4°C , 250g for 7 minutes. The supernatant was discarded and the cells resuspended in Medium 199 or Eagle's MEM without serum to the desired concentration. The cell count and viability of the suspension was determined by counting a 1/10 dilution in trypan blue (0.5% in physiological saline) in a haemocytometer (Improved Neubauer). One mouse usually yielded $4-6 \times 10^6$ peritoneal exudate cells; viability, as determined by the ability to exclude the trypan blue dye, always exceeded 95%.

8.2 In vitro culture of peritoneal exudate macrophages.

For the assay for binding of bacteria peritoneal exudate cell suspensions were adjusted to approximately 4×10^6 cells/ml and layered on to 7 x 20 mm "flying" coverslips in 12 x 74 mm glass tubes (W.R. tubes) in approximately 0.125 ml amounts with a pasteur pipette. The tubes were incubated at 37°C for $1\frac{1}{2}$ hours to allow the macrophages to adhere. Non-adherent cells were then removed by washing the coverslips thoroughly 7 times with Dulbecco's PBS + B and the remaining monolayer of adherent cells used in the experiment.

For the estimation of uptake of myo-(2- ^3H)-inositol the concentration of peritoneal exudate cells was adjusted to $2.5-3 \times 10^6$ cells/ml. The cell suspension was transferred into 13 x 150 mm glass culture tubes in 3 ml amounts, incubated on a slope for $1\frac{1}{2}$ hours at 37°C and washed free of non-adherent

cells as described above. The adherent cells were reincubated overnight in Eagle's MEM containing 5% heat-inactivated foetal calf serum before use in the experiment.

8.3 Counting of adherent cells.

The number of adherent cells was determined by inverting sample coverslips or coverslips prepared from aliquots of suspensions used for tube cultures on to a haemocytometer after they had been washed as described and overlaid with trypan blue. Two 1 mm squares were counted on each coverslip and the number of adherent cells per ml of the original cell suspension calculated by multiplying the number counted by 70 (to give the number per 140mm^2 coverslip) and 8 (to give the number per ml as each coverslip contains $1/8$ of a ml). Each coverslip as used for the binding assay contained approximately 2.5×10^5 adherent cells and each culture tube approximately 3×10^6 adherent cells. About one third of the peritoneal exudate cell population of normal mice was found to be adherent under the conditions described and the viability of the adherent cells was around 95%.

For comparative estimation of cell numbers in the culture tubes these were examined under an inverted microscope using an ocular grid.

8.4 Identification of macrophages.

A stock suspension of C. parvum was incubated with an equal volume of mouse anti-C. parvum antiserum (in a 1/15 dilution) for 30 minutes at 37°C and then diluted one in five in Eagle's MEM. The adherent cells on coverslips were incubated for 1 hour at 37°C with this suspension and then processed for microscopy as will be described below. Around 95% of the adherent cells showed vigorous phagocytosis of the antibody-coated particles and thus exhibited the two major defining characteristics of mononuclear phagocytes, adherence to a surface and efficient phagocytosis of antibody-coated particles (van Furth et al., 1972). These cells will hereafter be called macrophages.

9. BINDING OF BACTERIA TO MACROPHAGES AND INHIBITION OF BINDING

9.1 Assay for binding of micro-organisms to macrophage monolayers.

Macrophage monolayers on coverslips were overlaid with a suspension in Dulbecco's PBS + B of the appropriate bacteria at concentrations that had been found in preliminary experiments to result in binding of bacteria to approximately 30% of the macrophages. This degree of binding was found to be accompanied by minimal non-specific sticking of bacteria to the glass coverslip as well as little clumping of bacteria thus making counting easy and reproducible. The number of organisms required varied slightly for different bacterial species as will be shown

in the "Results". The tubes with coverslips were cooled on ice before adding the ice-cold suspensions of bacteria and the binding then allowed to take place at 4°C for 2 hours. In a few experiments using C. parvum the binding assay was performed at room temperature for 30 or 45 minutes. After this exposure to bacteria the coverslips were washed free of non-attached bacteria by thorough rinsing in the tubes 7 times with Dulbecco's PBS and shaking in 3 changes of Dulbecco's PBS. The coverslips were then air-dried, heat-fixed and mounted on to microscopic slides, cells facing up, with Harleco mountant. After the mountant had dried the coverslips were stained by the Gram method. The coverslips were examined microscopically under oil immersion and 1000x magnification. The percentage of cells with two or more attached organisms was determined, counting randomly a total of approximately 200 cells on each coverslip.

9.2 Inhibition of binding of bacteria to macrophage monolayers.

Macrophage monolayers on coverslips were overlaid with solutions in Dulbecco's PBS + B of various sugars, sugar derivatives and cell wall components prepared as described above. Preliminary experiments had shown that 10 mM was the minimum effective concentration of glucose and galactose that inhibited binding of C. parvum to the macrophages. This concentration, which is approximately twice the normal concentration of glucose in body fluids, was subsequently used for all sugars and sugar derivatives. The culture

supernatant and the acid and lipid extracts of C. parvum as well as lipid A were used without further dilution following preparation. These solutions were added ice-cold to the coverslips which had also been cooled on ice. The coverslips were then kept at 4°C for 20 minutes in order to allow the test substances to attach to the surface of the cells without internalization or metabolism. Following this the monolayers were washed gently 3 times with ice-cold Dulbecco's PBS + B and the binding assay performed at 4°C as described above.

9.3 The effect of divalent cations on binding of C. parvum to macrophage monolayers.

In order to chelate free divalent cations from bacterial suspensions and macrophage monolayers they were treated with EDTA. A stock suspension of C. parvum in physiological saline was mixed with EDTA to a final concentration of 10 mM EDTA, left at room temperature for 10 minutes, centrifuged for 20 minutes at 2500g and the supernatant discarded. Exposure to EDTA was repeated twice and the bacteria finally resuspended in Dulbecco's PBS without B. The macrophage monolayers were exposed to 10 mM EDTA in Dulbecco's PBS for 10 minutes at 37°C, washed twice with Dulbecco's PBS containing none or the appropriate known concentration of Ca^{++} - and Mg^{++} -ions and incubated with the same solution for 10 minutes at 37°C. Following this the monolayers were drained and the bacterial suspension added.

This was allowed to settle at room temperature for 15 minutes, the coverslips were drained gently and Dulbecco's PBS containing the appropriate concentration of Ca^{++} - and Mg^{++} -ions added. The coverslips were kept either for 2 hours at 4°C or 45 minutes at room temperature and then processed as described above.

10. TREATMENT OF MACROPHAGE MONOLAYERS WITH ENZYMES, CHEMICALS AND ANTISERUM

10.1 Treatment with enzymes.

Macrophage monolayers were exposed to the appropriate enzyme solution for 12 minutes at 37°C . These conditions had been found to be suitable in preliminary experiments using trypsin and to cause minimal damage to the cells. The highest non-toxic concentration for each enzyme was determined in preliminary experiments and used for subsequent experiments. Following exposure to the enzymes the tubes with coverslips were cooled on ice and washed 5 times with Dulbecco's PBS + B before performing the binding assay as described above.

10.2 Treatment with periodate and borohydride.

Sodium periodate (NaIO_4) and potassium borohydride (KBH_4) were used at a concentration of 1 mM in Dulbecco's PBS. The monolayers were incubated with either of these solutions for 5 minutes at 37°C and then washed 5 times with Dulbecco's PBS + B before use in the binding assay. These conditions were

shown by testing for trypan blue exclusion not to affect the viability of the cells.

10.3 Recovery from effects of enzymes or chemicals.

In order to study the recovery from the effects of treatment with enzymes or chemicals macrophage monolayers were incubated for 1 hour at 37°C with tissue culture medium (199 or Eagle's MEM) without serum after the enzymes or chemicals had been washed away, before adding the bacterial suspension. The role of divalent cations in this recovery was investigated by adding EDTA at a final concentration of 10 mM to the tissue culture medium.

10.4 The role of cell-bound antibody in the binding of C. parvum to macrophages.

In experiments testing for the role of cell-bound antibody in the binding of C. parvum to macrophages trypsin was used as described above at a concentration of 0.5 mg/ml and the monolayers incubated for 30 minutes at 37°C in Medium 199 after the enzyme had been washed away. Mouse anti-C. parvum antiserum was used at a dilution of 1/15 in Dulbecco's PBS + B and the macrophages incubated with this for 30 minutes at 37°C. The monolayers were then washed with Dulbecco's PBS and used in the binding assay or subjected to the treatment with trypsin as will be described in the "Results".

11. PREPARATION AND CULTURE OF SPLENIC LYMPHOCYTES

11.1 Preparation of spleen cells.

Spleens were taken from killed normal mice or mice that had been immunized 14 days previously with C. parvum. A suspension of spleen cells was prepared by pushing 1 or 2 spleens, previously cut into a few pieces, gently through a sterile wire mesh in a Petri dish containing Dulbecco's PBS. This suspension was centrifuged at 4°C for 7 minutes at 250g and red blood cells removed by resuspending the cells in the red blood cell lysing buffer described above for 4 minutes at room temperature. The cells were centrifuged again and finally resuspended by gentle pipetting in the appropriate tissue culture medium and the cell count adjusted for the intended use.

11.2 Preparation of lymphocyte culture supernatants.

For the production of lymphocyte culture supernatants the spleen cell suspensions were adjusted to a final concentration of 2×10^6 cells/ml in Eagle's MEM without serum after cell clumps had been allowed to settle out for a few minutes at room temperature. These suspensions were cultured for 4 hours at 37°C in 10 ml amounts in flat tissue culture bottles in the presence or absence of C. parvum at a final concentration of 2×10^8 bact./ml. The supernatants were harvested by centrifuging at 500g for 15 minutes at 4°C and centrifuging the

resulting supernatant again at 2000g for 20 minutes at 4°C. The supernatants were stored overnight at 0°C before use.

11.3 Separation of T-lymphocytes.

A cell population enriched in T-cells was prepared from spleen cell suspensions from normal mice or mice immunized 14 days previously with C. parvum using a method slightly modified from that of Julius et al. (1973) which was based on the observation that macrophages, polymorphonuclear leucocytes and B-lymphocytes tend to adhere to nylon wool whilst T-lymphocytes are non-adherent. Columns of nylon wool were prepared by packing 10 ml sterile plastic syringe barrels with sterile nylon wool from LP-1 Leuko-Pak Leukocyte Filters (Fenwal Laboratories, Morton Grove, Illinois) up to the 7 ml mark. The nylon wool was rinsed thoroughly with several changes of Dulbecco's PBS and left to soak overnight in Dulbecco's PBS at 4°C after closing the bottom by sticking the needle into a rubber stopper. The columns were then drained and rinsed and soaked with Eagle's MEM containing 5% heat-inactivated foetal calf serum (FCS) for 30 minutes at 37°C. A suspension of 5×10^7 to 10×10^7 spleen cells in 1 ml of Eagle's MEM with 5% FCS was layered on to the column and allowed to soak into it by draining 1-2 ml of medium and topping up again with warm medium. The column was incubated for 45 minutes at 37°C and the non-adherent cells then eluted with 10-15 ml of warm Eagle's

MEM with 5% FCS. The eluted cells (yield between 1×10^7 and 2×10^7) were centrifuged once at 250g for 10 minutes at room temperature and resuspended in Eagle's MEM without serum to the desired concentration. This procedure was reported by Julius et al. (1973) to result in a cell population consisting of well over 90% T-cells. In this laboratory, however, it has been found that T-cells were sometimes only 82% of the eluted cell population according to esterase staining (Mueller et al., 1975) the remainder consisting of B-cells (David Walkingshaw, personal communication).

11.4 Assay of DNA-synthesis by enriched T-cell population.

The effect of exposure to C. parvum in vitro on DNA-synthesis by T-lymphocytes from normal mice or mice immunized 14 days previously with C. parvum was estimated by measuring the uptake of tritiated thymidine (see Waithe & Hirschhorn, 1973). T-lymphocytes separated on a nylon wool column were incubated at a concentration of 2×10^6 cells/ml in flat bottom microtitre tissue culture plates (Sterilin, Teddington, Middlesex) in 0.2 ml of Eagle's MEM containing 10% heat-inactivated foetal calf serum and 0.04 mM 2-mercaptoethanol. The microtitre plates had been prepared by incubating them for $1\frac{1}{2}$ hours with normal mouse peritoneal exudate cells. The non-adherent peritoneal exudate cells had been washed away leaving the

macrophages in a final concentration of approximately 5% of the number of T-lymphocytes thus providing sufficient macrophages to enable the lymphocytes to respond to stimulation. C. parvum was added in 10 μ l amounts to give the final ratios of 100 and 500 bacteria per cell. These microtitre plates were incubated at 37°C for 3 days or 6 days. Four hours before the end of the culture period the medium was gently removed and replaced by the same volume of medium containing (6-³H)-thymidine, 0.2 μ Ci per well. The non-adherent cells were finally precipitated onto Whatman glass fibre filters with ice-cold 10% trichloroacetic acid in a sampling manifold (Millipore Corporation, Bedford, Massachusetts), see Freiesleben Sørensen et al. (1969). The filters were washed with a few ml of ethanol, air-dried and transferred into liquid scintillation counting vials. Five ml of scintillation fluid containing 5g/l of PPO (2,5-diphenyloxazole) and 0.12g/l of POPOP (1,4-bis-(2-(5-phenyloxazolyl))-benzene (both from Packard Instrument Company, Downers Grove, Illinois) in toluene (scintillator grade, Searle, Hopkins & Williams, Chadwell Heath, Essex) and radioactivity counted for 10 minutes in a Packard Scintillation Spectrometer.

The staff of the Virus Research Laboratory in the Department of Bacteriology kindly allowed the use of their sampling manifold and scintillation spectrometer.

12. UPTAKE OF MYO-(2-³H)-INOSITOL INTO MACROPHAGE PHOSPHATIDYLINOSITOL

12.1 Assay for uptake of myo-(2-³H)-inositol by macrophage monolayers.

Tube-cultures of macrophages were used prepared as described above. After the macrophage monolayers had been incubated overnight with Eagle's MEM with 5% foetal calf serum they were washed with Dulbecco's PBS + B and all subsequent steps performed in Eagle's MEM without serum. The cells were then incubated at 37°C with various test particles and substances in 3 ml volumes for different time period, as will be indicated in the "Results" but usually for 4 hours. One hour before the end of the culture period 2 µCi of myo-(2-³H)-inositol were added to each tube in 0.1 ml of Dulbecco's PBS. The cell metabolism was stopped by placing the tubes into ice. The monolayers were then washed 7 times with cold Dulbecco's PBS and 5 ml of 2:1 chloroform-methanol added to each tube. The lipids were extracted for 20 minutes at 37°C with vigorous shaking on a Rotamixer every 5 minutes. Two ml of liver lipid extract (prepared as described above) were added as a carrier to each tube with 3 ml of 0.5 M MgCl₂ and mixed well. Preliminary experiments had shown that the addition of a lipid carrier greatly reduces the loss of labelled lipid during the extraction and separation procedure and thus improves reproducibility. The phases were separated by centrifugation, the aqueous phase

discarded and the organic phase equilibrated once more against 0.5 M MgCl_2 but without mixing this time. After the water-soluble phase had been carefully removed with a pasteur pipette the organic phase was transferred quantitatively into liquid scintillation vials taking care not to carry over residual droplets of the aqueous phase. The lipid was evaporated to dryness at 40°C under a stream of N_2 . In preliminary experiments anhydrous Na_2SO_4 had been used to absorb residual water from the organic phase but it was soon found that if small amounts of this salt contaminated the lipid phase on transfer into the liquid scintillation vials this resulted in a colour reaction with the scintillation fluid and thus interfered with the counting of radioactivity. Treatment with anhydrous Na_2SO_4 was therefore omitted in all subsequent experiments and no problems resulting from possible contamination with the water-soluble phase were encountered. The dried extract was finally redissolved in 2 ml of toluene-based scintillation fluid containing 5 g of PPO and 0.13 g of POPOP per litre as above and radioactivity counted for 10 minutes in a Packard Scintillation Spectrometer. Counting was repeated three times in order to ensure that counts were stable and there was no non-specific interference with the scintillation fluid. Refrigeration during counting was found to be essential, higher temperatures caused fluctuations in the counts.

Radioactivity of samples of the myo-(2-³H)-inositol solution added (10 μ l) and culture medium, washings and aqueous phases collected at the end of the experiment (100 μ l) was counted in a few experiments, using scintillation fluid containing equal parts of toluene and Triton X-100 (scintillator grade, Searle, Hopkins & Williams, Chadwell Heath, Essex) and 8g/l of PPO.

12.2 Identification of phosphatidylinositol by thin layer chromatography.

In order to ensure that the radioactivity measured in the lipid extract was solely associated with phosphatidylinositol the phospholipids of a lipid extract from macrophages that had been incubated for two hours with myo-(2-³H)-inositol were separated by thin layer chromatography using the method of Skipski et al. (1964). Silica gel H (Stahl type 60, Merck, Darmstadt, Germany) was slurried with water and applied at a thickness of 0.25 mm on to 5 x 20 cm glass plates. The plates were allowed to dry at room temperature for 20 minutes and were then activated just before use at 100°C for 1½ hours. The lipid extract and reference samples of phosphatidylinositol (Koch Light Laboratories, Colnbrook, Buckinghamshire) and phosphatidylcholine (kindly provided by Dr. Keith Suckling, Department of Biochemistry, University of Edinburgh) were spotted on to the

plates and the chromatograms developed with a solvent system consisting of chloroform-methanol-acetic acid-water (25:15:4:2 by volume). The plates were then air-dried and the lipids visualized by spraying with phosphomolybdic acid or exposure to iodine vapour. The separated lipids were scraped off the plates and transferred into liquid scintillation vials and radioactivity counted as described before.

13. TESTS FOR MACROPHAGE EFFECTOR FUNCTIONS

13.1 Assay for bacteriostatic activity of macrophages against *Listeria monocytogenes*.

An estimation of relative bacteriostatic activity against *L. monocytogenes* was used to indicate whether the macrophages had become activated. An in vitro assay was adopted from Ruskin et al. (1969) using coverslip cultures of macrophages prepared as described above. The coverslips were incubated with *E. coli* lipopolysaccharide ($60 \mu\text{g}/10^6$ cells) in Eagle's MEM without serum for 4 hours or 24 hours. The cells were then washed three times with Dulbecco's PBS + B and overlaid with a suspension of live *Listeria monocytogenes* in Eagle's MEM containing 15% heat-inactivated foetal calf serum but no antibiotics to give a ratio of 7-10 bacteria per cell. The coverslips were incubated for 30 minutes at 37°C to allow phagocytosis to take place after which free bacteria were removed by washing 6 times with Dulbecco's PBS + B. Half of

the coverslips were then air-dried, heat-fixed and Gram-stained for microscopic examinations, as described above, to determine the degree of phagocytosis. The remaining half of the coverslips was reincubated with Eagle's MEM with 15% heat-inactivated foetal calf serum and no antibiotics for 4 hours at 37°C (in some experiments penicillin and streptomycin were added, 5 µg/ml of each which is 1/10 of the usual tissue culture medium concentration). The coverslips were then processed in the same way as described before. The number of cells per microscopic field, the number of cells with ingested bacteria and the number of intracellular bacteria were counted; 12-18 fields were counted on each coverslip. There were few extracellular bacteria and no obvious extracellular multiplication of bacteria.

13.2 Estimation of intracellular and extracellular lysosomal enzymes.

The effect of various stimuli used in the assay for phosphatidylinositol turnover on the synthesis and secretion of 3 lysosomal enzymes by macrophages was tested. Tube cultures of macrophages were prepared as described above and incubated for 6, 24 or 48 hours with the appropriate stimulant in Eagle's MEM without serum. At the end of each culture period the culture supernatants were removed and assayed for enzyme activity. In order to estimate intracellular concentrations of the enzymes the cells were lysed by the addition of 3 ml of distilled water

and rapid freezing in liquid N₂ alternated with thawing at 37°C, three times.

The enzyme assays were performed as follows:

β -galactosidase was measured by incubating 0.5 ml of the sample with 0.5 ml of 2.5 mM p-nitrophenyl- β -D-galactopyranoside (Koch Light Laboratories, Colnbrook, Buckinghamshire) in 3 ml of 0.1 M citric acid buffer, pH 3.6 for one hour at 37°C. The reaction was stopped by adding 4 ml of 0.4 M glycine buffer, pH 10.8. If any precipitate formed this was removed by centrifugation at 1500g for 20 minutes. The optical density of the clear supernatant was read at 430 nm (Method of Couchie *et al.*, 1959).

For the estimation of β -glucuronidase 0.5 ml of the sample were incubated for one hour at 37°C with 0.5 ml of 0.01 M phenolphthalein glucuronic acid (rabbit urine, Sigma Chemical Company, St. Louis, Missouri) in 4 ml of 0.1 M acetate buffer, pH 4.5. The reaction was stopped by adding 5 ml of 0.4 M glycine buffer, pH 10.8 and the optical density read at 540 nm (From Talalay *et al.*, 1946).

The concentration of acid phosphatase was assayed by incubating 0.5 ml of the sample with 0.1 ml of 2.5 mM p-nitrophenyl-phosphate (Sigma Chemical Company, St. Louis, Missouri) and 0.25 ml of 0.4 M acetate buffer, pH 5.0. The reaction was stopped by the addition of 4 ml of absolute ethanol. The colour change was facilitated by adding 0.4 ml of 0.25 N NaOH and the optical density read at 410 nm. (Method developed in

this laboratory by Dr. Bill McBride and Sheena Tuach).

Reference enzymes were obtained from the following sources:

β -galactosidase (bovine liver) and acid phosphatase (type II, potatoes) from Sigma Chemical Company, St. Louis, Missouri;

β -glucuronidase (molluscs) from British Drug Houses, Poole, Dorset.

14. CALCULATIONS AND STATISTICAL METHODS

Experimental results varied somewhat between individual experiments. This was caused mainly by some variability between different batches of bacteria and loss of cell wall material during storage as well as variations in the mice and differences in the numbers of cells obtained. Relative values showed, however, good agreement between experiments. In order, therefore, to enable better comparison between experiments and make the pooling of results from separate experiments possible the experimental data were often converted into relative values thus: $\frac{t}{c} \times 100$, where t is the measurement obtained under the test conditions and c is the averaged measurement of the untreated controls in the same experiment. The value for untreated controls, therefore, becomes 100. Calculated standard errors of the percentage (see below) in the binding and inhibition assays were converted in the same way, $\frac{\text{calculated SE}}{c} \times 100$. Results for binding assays and inhibition experiments for one type and batch of organisms usually showed only variation within the standard error and could thus be pooled without conversion

but expression in relative values makes comparisons between inhibition experiments using different types of bacteria easier.

The results of the bacteriostatic assay were treated as follows: the number of intracellular bacteria per total number of cells was calculated from the counts obtained as this takes account of the proportion of cells containing bacteria. This figure was used to calculate the intracellular growth ratio of L. monocytogenes by dividing the number of bacteria per cell at 4 hours by the number (bact./cell) directly after phagocytosis. The relative bacteriostatic effect of lipopolysaccharide-treated macrophages compared with controls was calculated as: (growth ratio in controls/growth ratio in treated cells) multiplied by 100 to obtain the percentage value.

For the results of binding assays and inhibition experiments the standard error of the percentage was calculated according to the formula: $\frac{p \times (100-p)}{n}$, where p is the observed percentage and n is the number of individuals (Bradford Hill, 1967). Here p was the percentage of cells with attached bacteria and n the total number of cells counted to determine that percentage.

For pooled results of experiments on the uptake of myo-(2-³H)-inositol standard errors of means of data converted into relative values were calculated as: $\frac{\text{standard deviation}}{n}$ where n is the number of observations and the standard deviation by the

formula: $\sqrt{\frac{\sum (x-\bar{x})^2}{(n-1)}}$, x stands for individual values (Bradford Hill, 1967). Results from experiments on the uptake of tritiated thymidine by lymphocytes showed sufficiently small variation to be pooled directly and statistical calculations were performed on the unconverted data.

Probability values (p values) were obtained from Geigy Scientific Tables using two-tailed Student's t-test. The t value was calculated according to the formula: $\frac{\bar{x}_1 - \bar{x}_2}{\text{SED}}$ where \bar{x}_1 and \bar{x}_2 are the two means to be compared and SED is the standard error of the difference calculated thus: $\frac{s^2}{n_1} + \frac{s^2}{n_2}$ where n_1 and n_2 are the numbers of observations behind each of the means and s^2 is derived from the individual standard errors of the means, s_1 and s_2 by the formula: $s^2 = \frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2}$

RESULTS

RESULTS

1. NON-SPECIFIC RECOGNITION BY MACROPHAGES

The first part of this study was concerned with non-specific recognition by macrophages. This chapter describes the binding of non-opsonized bacteria to mouse macrophages and the inhibitory effects of various sugars on such binding. The two following chapters deal with the requirement for divalent cations for the binding of C. parvum and the results of experiments using enzyme and chemical treatment of macrophages in an attempt to elucidate the nature of the plasma membrane components involved in the binding of bacteria.

1.1 Binding of different types of non-opsonized bacteria to mouse peritoneal exudate macrophages at 4°C.

In a series of initial experiments the ability of several different types of bacteria to bind to macrophage monolayers on coverslips in the absence of serum was studied. The aim was to show whether binding occurred and to establish the concentration of each type of micro-organism that would be suitable for use in subsequent inhibition assays. It was also necessary to test whether the formalin-killing had an effect on the binding. The results of these experiments are summarized in Table 1. It can be seen that only two out of eleven different species of bacteria tested show very little binding, viz. Streptococcus

Table 1:

Binding of live and formalin-killed bacteria to mouse peritoneal macrophages at 4°C

Organism	Live bacteria		Formalin-killed bacteria	
	No. /ml	Percentage of macrophages binding bact.	No. /ml	Percentage of macrophages binding bact.
<u>Str. viridans</u>	5×10^9	34.0	5×10^9	33.7
<u>Str. pyogenes</u>	4×10^9	21.7	5×10^{10}	23.5
	4×10^8	15.0	5×10^9	18.8
			5×10^8	16.0
<u>Str. pneumoniae</u>	2.5×10^{10}	5.7	(6×10^{11})	27.7)*
			2.5×10^{10}	7.0
<u>B. anthracoides</u>	3×10^9	23.5	3×10^{10}	47.0
	3×10^8	11.5	3×10^9	25.5
			3×10^8	8.5
<u>St. albus</u>	5×10^9	28.5	7×10^{10}	47.0
	2×10^9	7.7	7×10^9	23.8
			2×10^9	3.8
			2×10^8	0.7
<u>St. aureus</u>	2×10^9	22.3	2×10^9	22.8
	5×10^8	7.0	3×10^8	13.2
			8×10^7	5.3
<u>C. parvum</u> 10390			3×10^{10}	14.4
			6×10^9	14.1**
<u>P. freudenreichii</u>			3×10^{10}	2.7
<u>E. coli</u>	3×10^9	not countable	(7×10^9)	74.0)*
	5×10^8	26.0	7×10^8	29.0
			7×10^7	8.5
<u>K. aerogenes</u> (teaching collection)	2×10^9	18.5	1×10^{10}	24.0
			1×10^9	16.5
			5×10^8	8.5
<u>K. aerogenes</u> Wild type 5055			2×10^{10}	19.0
			2×10^9	11.3
<u>K. aerogenes</u> Non-capsulated mutant M2			1×10^{10}	16.7
			1×10^9	8.7
<u>K. aerogenes</u> Core mutant M 10B			2×10^{10}	18.5
			2×10^9	14.0
<u>Ps. aeruginosa</u> (mucoid)	2.5×10^{10}	65.0	1.5×10^{10}	20.8
	2.5×10^9	29.3		
<u>Ps. aeruginosa</u> (non-mucoid)	7×10^{10}	not countable	1×10^{10}	22.5
	7×10^9	19.5		
<u>S. typhimurium</u> SL 1542 (smooth)			1.5×10^{10}	not countable
			1.5×10^9	20.3
<u>S. typhimurium</u> SL 1096 (rough)			4×10^{10}	not countable
			4×10^9	32.0
<u>S. typhimurium</u> SL 1099 (rough)			1.2×10^{10}	not countable
			1.2×10^9	24.5
<u>S. typhimurium</u> SL 1102 (rough)			2×10^{10}	not countable
			2×10^9	30.5

*: Slides covered in bacteria, binding to macrophages not easily distinguishable

**: Subsequent batches of C. parvum showed higher degree of binding.

pneumoniae and Propionibacterium freudenreichii. In the case of Str. pneumoniae this may be caused by its capsule which is known to interfere with phagocytosis (Dubos, 1945). The capsule of the wild type of Klebsiella aerogenes (NCTC 5055) clearly had no inhibitory effect on binding. P. freudenreichii is related to Corynebacterium parvum (which may belong to the Propionibacterium acnes group according to the classification of Johnson & Cummins, 1972) and might thus have been expected to bind. When the experiments were performed this organism had been kept longer than was usual for the other bacteria and may have lost some of its cell wall material during storage. This was shown to happen with C. parvum, resulting in loss of Gram-staining characteristics and release of cell wall antigen into the suspending medium.

The table shows clearly that there was usually no difference in binding of living or dead organisms of the same type. As killed organisms are more convenient to work with they were employed in all subsequent experiments.

The concentration of organisms required to achieve similar degrees of binding varied somewhat between different species but was usually between 10^9 and 10^{10} bacteria/ml. The degree of binding was usually directly related to the concentration of bacteria although in the case of Str. pyogenes the differences were not very marked for different concentrations.

It was found that binding to more than approximately 30%

of the macrophages coincided with a higher degree of non-specific sticking of bacteria to the coverslips. For ease of counting and reproducibility bacterial concentrations that yielded 20-30% binding were, therefore, selected for use in the binding inhibition studies.

1.2 Effect of temperature, cytochalasin B and glutaraldehyde-fixation on binding and phagocytosis of *Corynebacterium parvum*.

The binding assay using *C. parvum* was performed at three different temperatures, 4°C, 20°C (room temperature) and 37°C in the presence or absence of cytochalasin B, a drug that interferes with microfilament function and inhibits phagocytosis (see "Introduction" 9.2.2.). The effect on binding of chemical fixation of the macrophages was also studied.

With increased temperature, binding of *C. parvum* took place more rapidly and was more marked (see Tables 2 and 3). The pattern of binding was also changed at higher temperatures as compared with 4°C. At 4°C only binding to the periphery of the cells was observed (see Figure 2). This was also the pattern seen for the first 20 minutes at room temperature (Figures 3 and 4). If binding was allowed to take place at room temperature for more than 20 minutes binding to the whole exposed surface of the cells, often in crescents giving a "hairy" appearance (see Figures 5, 6 and 7) became increasingly common and after 60-120 minutes phagocytosis was clearly noticeable

Table 2:

Effect of temperature and Cytochalasin B on binding of C. parvum to mouse peritoneal macrophages.

Temperature	Incubation time in minutes	Percentage of macrophages binding bacteria after exposure to:			
		<u>C. parvum</u> alone	SE	<u>C. parvum</u> + Cytochalasin B	SE
4°C	120	20.6	2.0	19.5	1.9
20°C	45	39.0	2.4	24.2	2.2
20°C	90	52.8	2.6	33.2	2.3
37°C	30	33.1*	2.3	22.8*	2.1

*: C. parvum alone: 58.5% of bound bacteria intracellular; no phagocytosis observed in presence of Cytochalasin B. One representative experiment.
SE: standard error of the percentage.

Table 3:

Effect of temperature and fixation with glutaraldehyde on binding of C. parvum to mouse peritoneal macrophages.

Temperature	Incubation time in minutes	Percentage of macrophages binding bacteria:			
		Macrophages unfixed	SE	Macrophages fixed with glutaraldehyde	SE
4°C	120	14.3	1.7	15.3	1.8
20°C	20	16.8	1.8	17.9	1.9
37°C	20	33.0*	2.4	15.3*	1.8

*: Unfixed macrophages: 61.3% of bound C. parvum intracellular; no phagocytosis by glutaraldehyde-fixed macrophages. One representative experiment.
SE: standard error of the percentage.

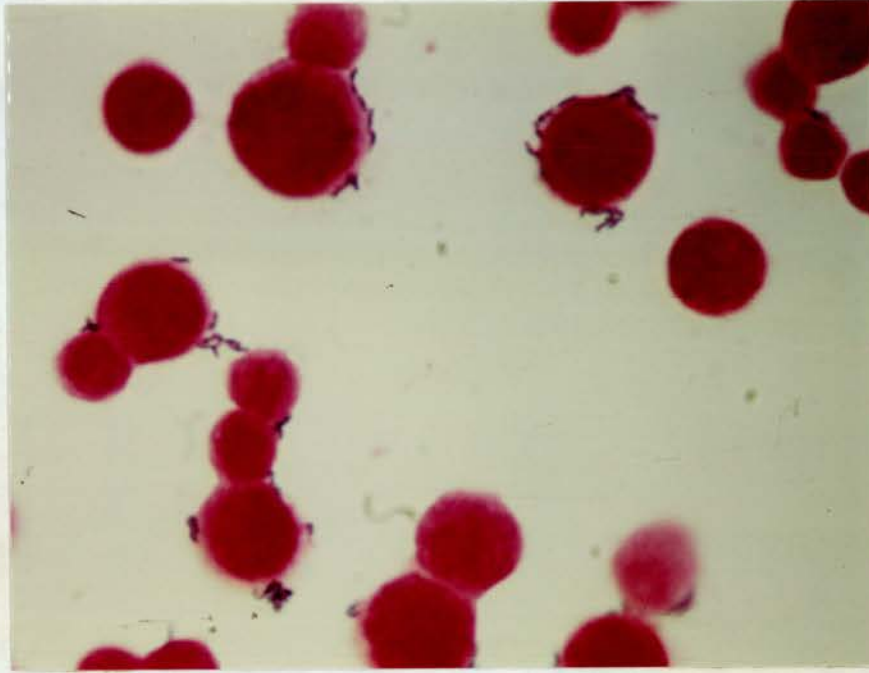


Figure 2. C. parvum binding to mouse macrophages after incubation for 2 hours at 4°C. .

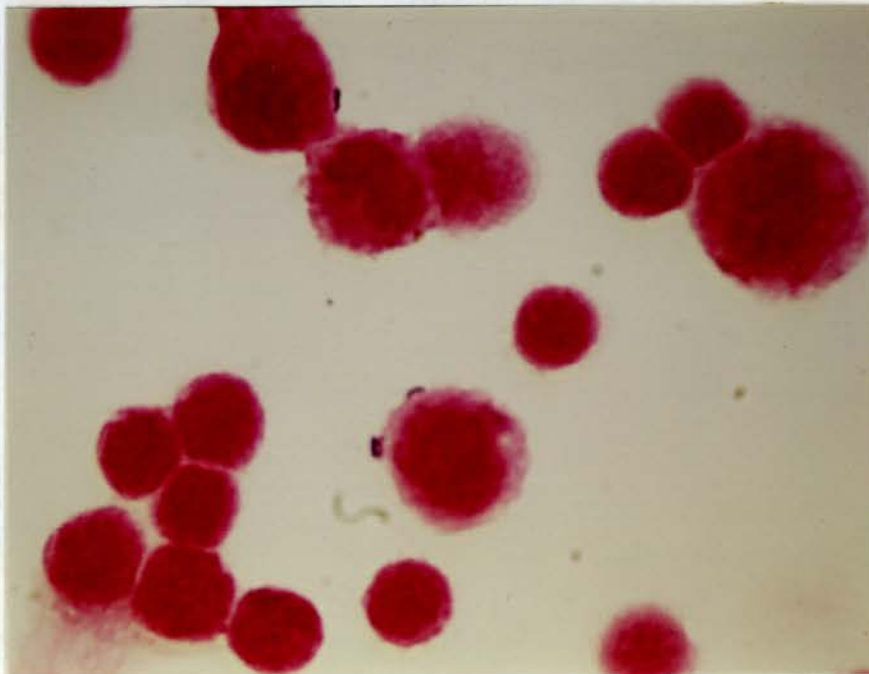


Figure 3. C. parvum binding to mouse macrophages after incubation for 10 minutes at 20°C.

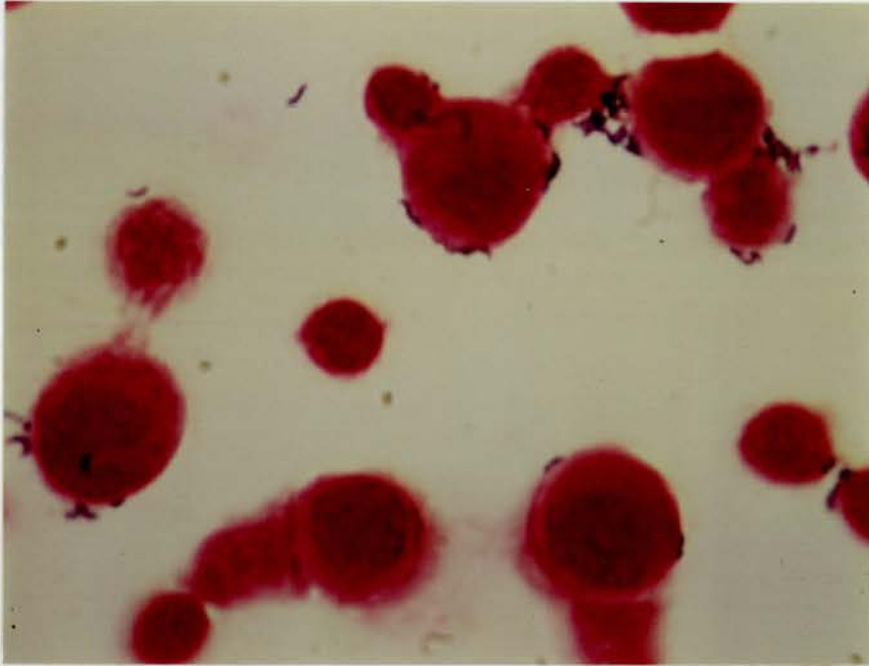


Figure 4. C. parvum binding to mouse macrophages after incubation for 20 minutes at 20°C.

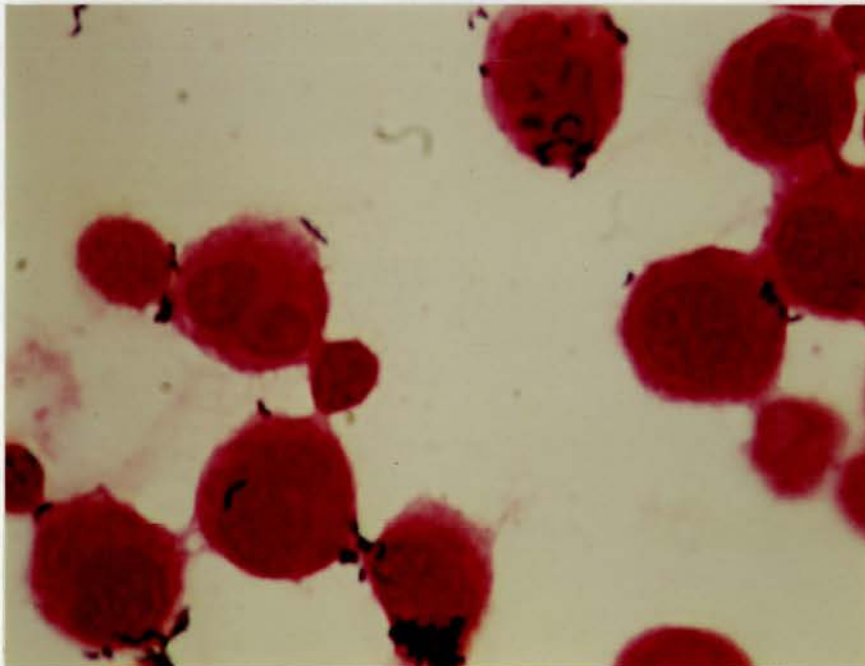


Figure 5. C. parvum binding to mouse macrophages after incubation for 45 minutes at 20°C.

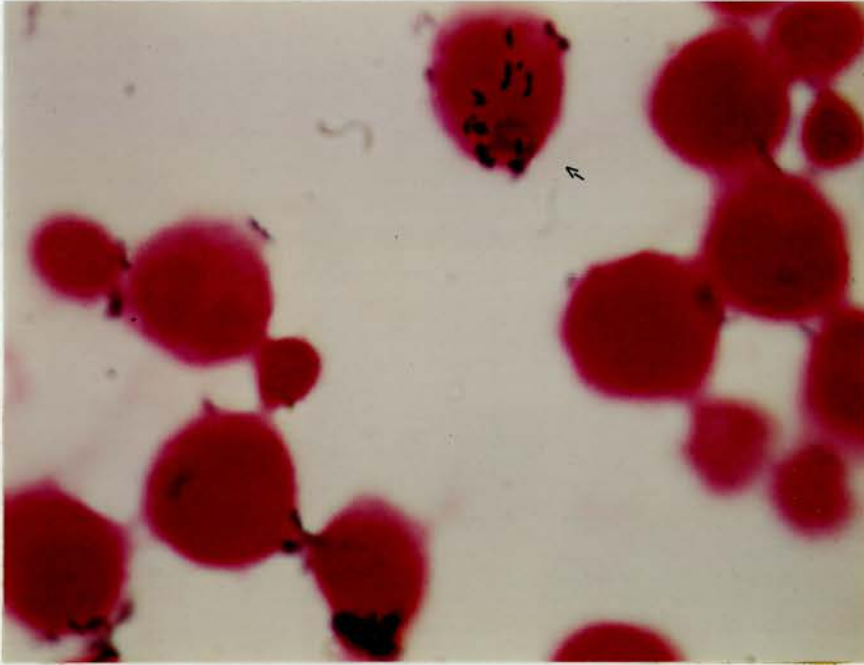


Figure 6. Same microscopical field as shown in Figure 5 with the focus changed to show bacteria attached to the exposed surface of the cell at the top.

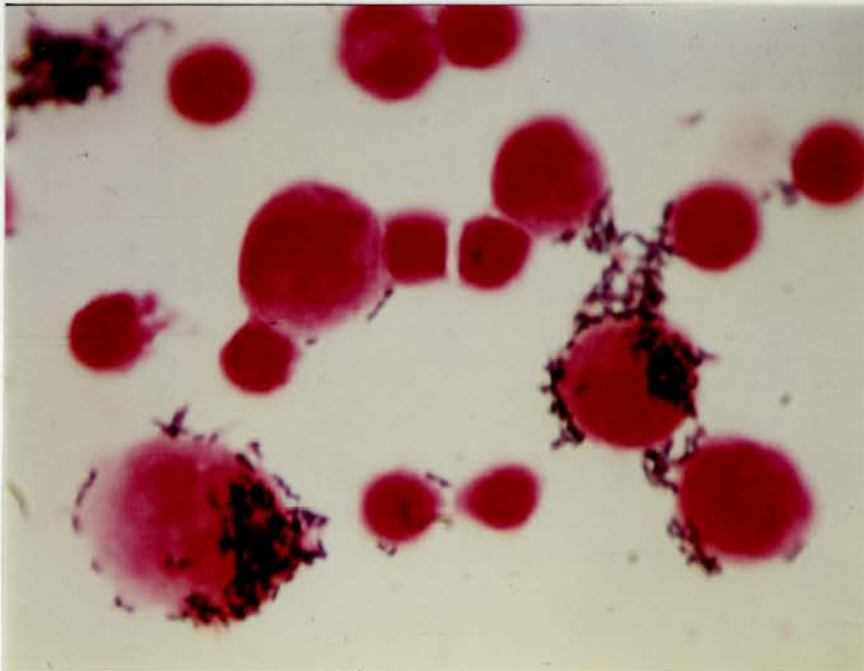


Figure 7. Binding and phagocytosis of C. parvum by mouse macrophages after incubation for 1 hour at 20°C.

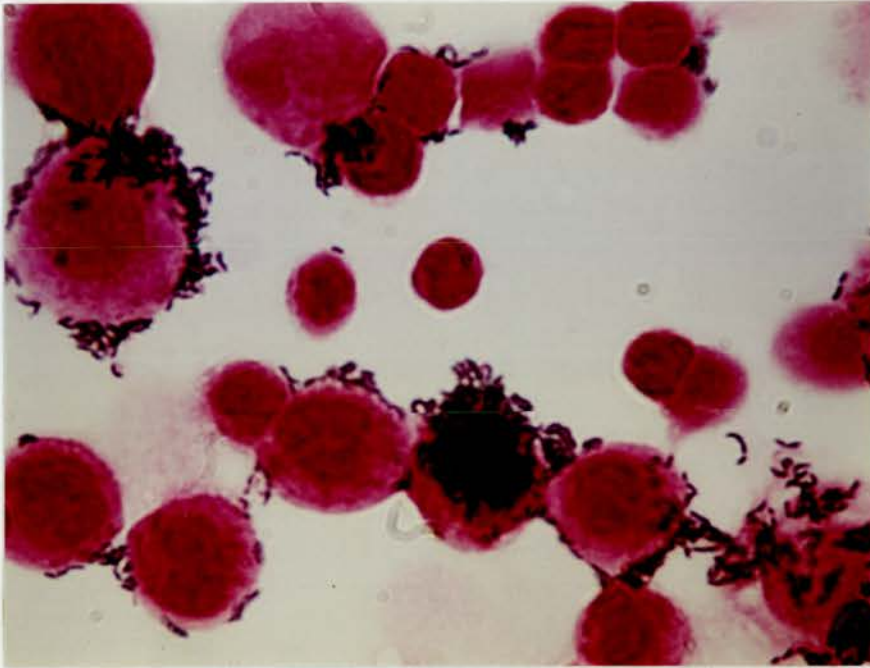


Figure 8. Binding and phagocytosis of C. parvum by mouse macrophages after incubation for 2 hours at 20°C.

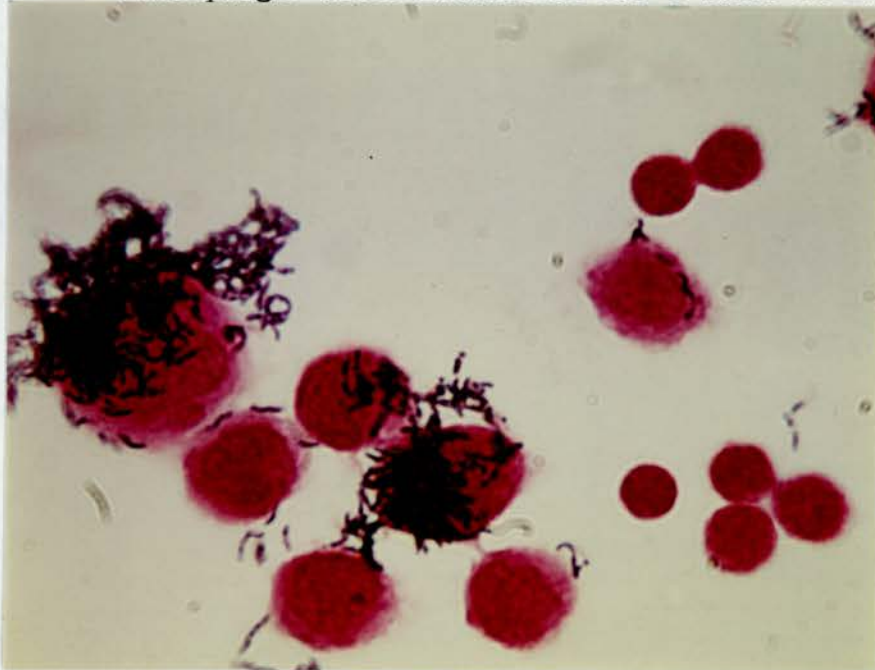


Figure 9. Phagocytosis and binding of C. parvum by mouse macrophages after incubation for 20 minutes at 37°C.

(Figures 7 and 8). At 37°C phagocytosis was marked within the first 30 minutes (Tables 2 and 3, Figure 9). If the cells were fixed with glutaraldehyde or exposed to C. parvum in the presence of cytochalasin B binding still took place (Tables 3 and 2) but there was no change in pattern of binding with increased temperature and no phagocytosis occurred (Figure 10). Increase in temperature also had a less marked effect

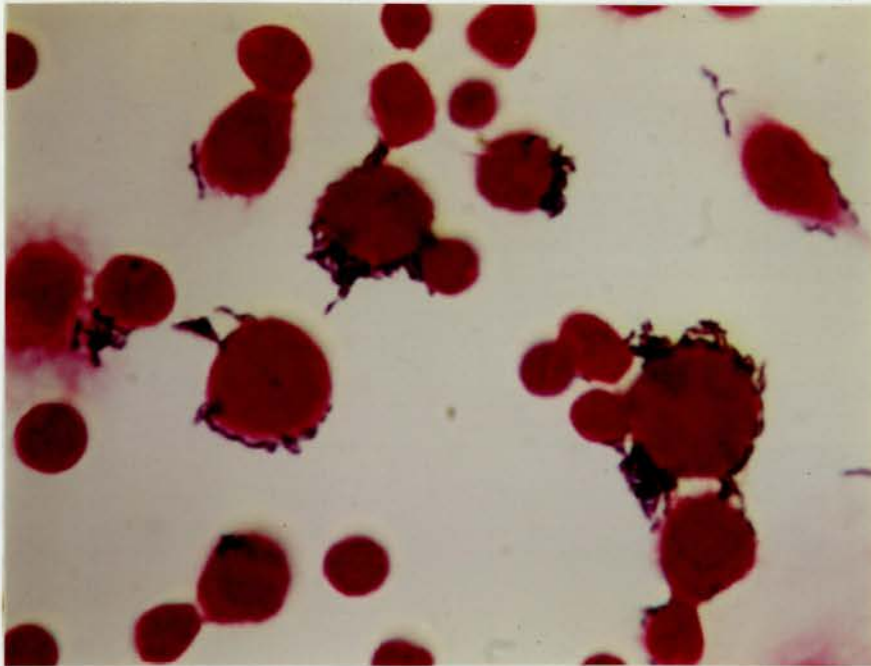


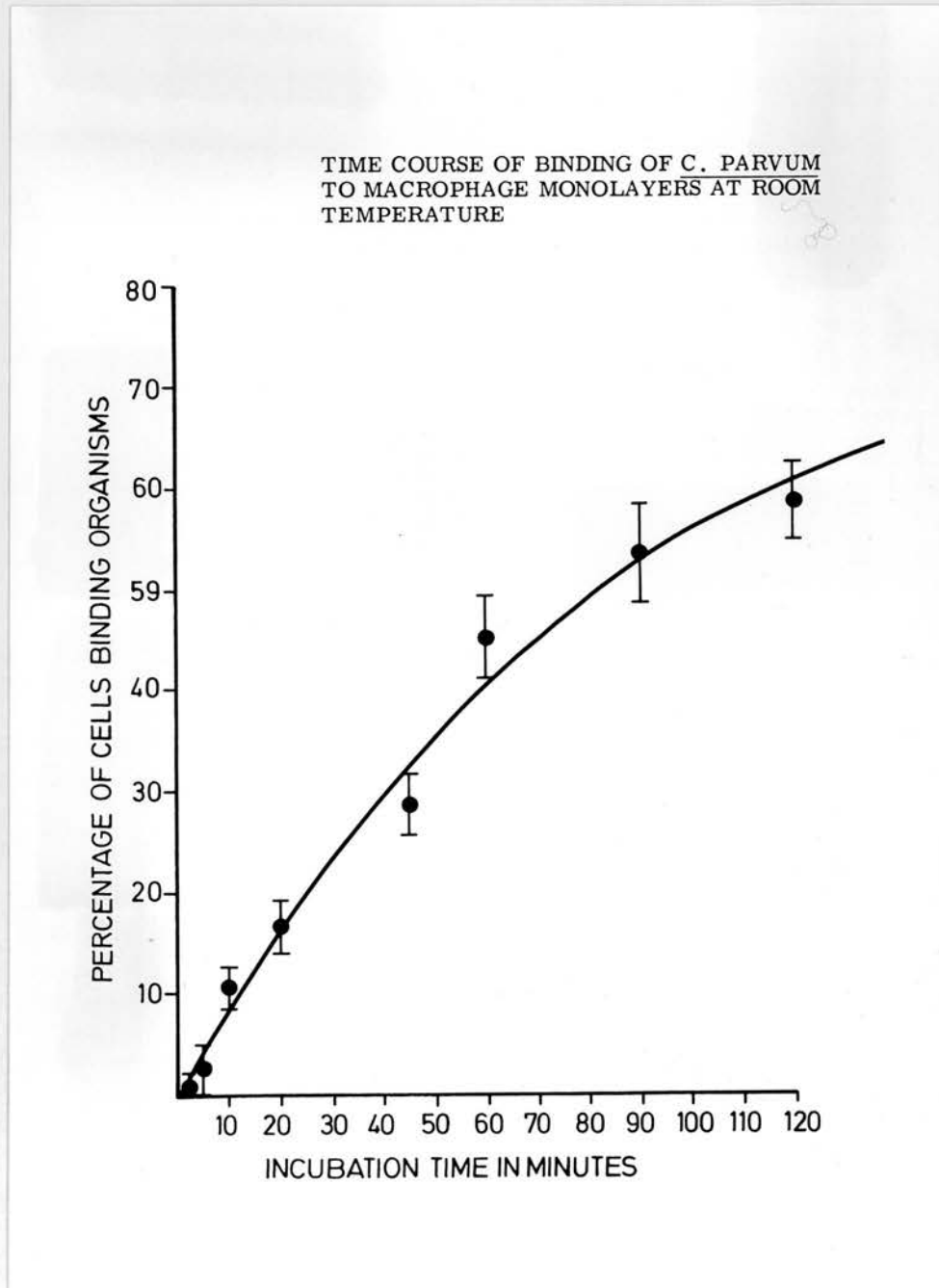
Figure 10. C. parvum binding to glutaraldehyde-fixed mouse macrophages after incubation for 1 hour at 20°C.

on the degree of binding when cytochalasin B was added (Table 2). These results indicate that the binding observed at 4°C was a passive phenomenon that did not involve cellular metabolism or participation of cellular motility. The changed pattern of

binding to untreated cells at the higher temperatures thus appears to be caused by movement of surface components. Phagocytosis is known to require microfilament function as mentioned above.

The time course of binding of C. parvum at room temperature is shown on Figure 11. It can be seen that the rate of binding

Figure 11.



slowed down after the first hour. This raises the question whether the cell population contains both binding and non-binding cells or cells with different rates of binding. It was observed that the macrophages on the monolayers could clearly be divided into large and small cells and in later experiments differences between these two cell types in their degree of phagocytosis were noted. Table 4 shows that the macrophage population was composed of approximately equal proportions of large and small cells and that the small cells were less active in binding and phagocytosing C. parvum.

Table 4:

Phagocytosis of C. parvum by large and small mouse peritoneal macrophages.

	Percentage of adherent cell population	Percentage of cells phagocytosing <u>C. parvum</u>
Large adherent cells	53.1)	58.8 SE: 2.9
Small adherent cells	46.9) SE: 2.2	8.9 SE: 1.8

C. parvum: 10^8 per ml, incubation for 4 hours at 37°C in serum-free medium.

Phagocytosis of bound bacteria: 99%. Pooled data from two separate experiments

SE: standard error of the percentage

These experiments did not distinguish between binding and phagocytosis since phagocytosis of bound bacteria was virtually complete after 4 hours, the incubation period used.

1.3 Role of cell-bound antibody in the binding of *C. parvum* to macrophages.

Many animal species including mice have serum antibodies that react with antigens of anaerobic coryneforms (Woodruff et al., 1974; McBride et al., 1975). It was, therefore, necessary to investigate the possibility that binding of *C. parvum* to macrophages might be mediated by cell-bound antibody. The mice used in the present experiments did not have *C. parvum*-reactive antibodies in their serum, as judged by agglutination of *C. parvum*-coated latex particles (titre $\leq 1/6$).

The effect of mild treatment of the macrophage monolayers with trypsin (0.5mg/ml for 12 minutes at 37°C) on the binding of *C. parvum* was studied. Trypsin is known to digest immunoglobulins (Stanworth & Turner, 1973). Following incubation with trypsin the cells were washed and reincubated in tissue culture medium for 30 minutes at 37°C in order to allow regeneration or repair of plasma membrane constituents that might have been affected by the trypsin treatment. It had been shown by Ilgen & Burkholder (1974) that a C4-determinant on the macrophage plasma membrane which is removed by trypsin is regenerated within minutes after removal of trypsin and later experiments in the present study were to show that although

treatment with a higher concentration of trypsin affected the ability of macrophages to bind C. parvum full binding capacity was regained within 1 hour at 37°C in tissue culture medium (see later).

It can be seen from Table 5 that the described treatment with trypsin had no effect on binding of C. parvum to the macrophages. Macrophages were also coated with specific mouse anti-C. parvum antibody by incubation at 37°C for 15 minutes. The antiserum, which had a latex agglutination titre of 1/384, was used at a dilution of 1/15. Coating of the macrophages

Table 5:

Effect of pre-treatment with trypsin and specific antiserum on the binding of C. parvum to mouse peritoneal macrophages.

Macrophage monolayers pre-incubated with:	Percentage of cells binding <u>C. parvum</u>			
	Experiments I & II	SE	Experiment III	SE
Dulbecco's PBS + B	12.8	1.8	55.3	2.2
Trypsin 0.5 mg/ml	11.2	1.4	55.4	2.1
Mouse anti- <u>C. parvum</u>	20.1	1.9	90.2	1.5
Mouse anti- <u>C. parvum</u> followed by trypsin 0.5 mg/ml	10.7	2.1	50.5	2.0
Normal guinea pig serum	13.8	1.4	not done	
Normal guinea pig serum followed by trypsin 0.5 mg/ml	10.7	2.1	not done	

The binding assay was performed at room temperature for 30 minutes in experiments I and II and 45 minutes in experiment III. In experiment III a newly grown batch of bacteria that showed unusually high binding ability was used whilst in experiments I and II an older stock suspension of bacteria was used. SE: standard error of the percentage.

with the specific antiserum led to a marked increase in the binding of C. parvum. Subsequent treatment of antibody-coated cells with trypsin in the way described above brought the binding ability down to background levels illustrating that the exposure to trypsin could destroy cell-bound antibody. The effect of the mouse anti-C. parvum antiserum was likely to have been specific as pre-incubation with normal guinea pig serum had no effect on the binding.

1.4 Inhibition of binding of several types of bacteria to macrophages by glucose and galactose.

Glucose and galactose at a concentration of 10 mM were shown to be potent inhibitors of binding of C. parvum to macrophages in preliminary experiments. These two sugars and their derivatives are found in the cell walls and capsules of many different types of bacteria (Salton, 1964, pp. 265-283) and were, therefore, used in a series of binding inhibition experiments performed as described in "Materials and Methods". It can be seen from Table 6 that each of these two sugars caused significant inhibition of binding of a wide range of bacteria. There were a few exceptions; binding of B. anthracoides was not inhibited by either glucose or galactose and preincubation with galactose also failed to inhibit binding of K. aerogenes (NCTC 5055), Str. viridans and Str. pyogenes. The results indicate that glucose and galactose are widely involved in the

Table 6:

Inhibition of binding of bacteria to mouse peritoneal macrophage
by glucose and galactose.

Organism	Binding (expressed as percentage of control value) to macrophages pre-incubated with:				Binding to untreated controls = 100% SE
	Glucose 10mM	SE	Galactose 10mM	SE	
<u>Str. viridans</u>	56.7	3.9	95.8	4.5	3.0
<u>Str. pyogenes</u>	49.2	5.1	97.9	6.7	4.1
<u>B. anthracoides</u>	96.5	7.4	107.8	5.4	3.5
<u>St. albus</u>	53.3	5.3	53.3	5.3	4.7
<u>St. aureus</u>	42.7	5.8	56.4	6.6	4.6
<u>C. parvum</u>	24.7	3.9	26.8	3.8	6.5
<u>E. coli</u>	47.6	5.8	51.5	5.2	4.8
<u>K. aerogenes</u> (WT 5055)	55.2	5.9	92.3	5.9	3.3
<u>Ps. aeruginosa</u> (mucoid)	53.3	5.5	47.8	10.0	5.0
<u>Ps. aeruginosa</u> (non-mucoid)	49.8	5.1	51.7	6.1	4.8
<u>S. typhimurium</u> (SL 1542)	51.6	4.8	52.8	4.8	2.6

Each figure represents the mean of pooled data from at least two separate experiments each performed in duplicates. SE: standard error of the percentage converted into percentage of control binding value (see Materials and Methods).

recognition of bacteria by macrophages. The exceptions demonstrate that bacterial binding can not be solely mediated by these two sugars, and this is also suggested by the observation that even when inhibition occurs the reduction in binding is usually only about 50% as compared with the untreated controls. The binding of bacteria that do not contain a particular sugar in their cell walls or express it freely on the surface would not be expected to be inhibited by previous binding of that sugar to the macrophage surface. Galactose appears to be a less common component than glucose of streptococcal cell walls (Salton, 1964, p. 172). K. aerogenes

has a capsule which contains glucose but no galactose (Poxton & Sutherland, 1976). Bacillus-species contain glucose in their teichoic acids (Salton, 1964, p. 161) but it would appear from the present data that this is not accessible for binding reactions. Staphylococci contain glucose and galactose in their cell walls (Salton, 1964, pp. 267-271). Pseudomonas aeruginosa contains glucose and galactosamine in its lipopolysaccharide (Meadow, 1975). No difference was observed in the binding or inhibition of binding between the mucoid and non-mucoid strain of Ps. aeruginosa. It was not realized at the time of these experiments that the culture conditions used did not favour the production or retention on the bacterial surface of the mucoid material (Govan & Fyfe, personal communication). Glucose and galactose are both found in the lipopolysaccharides of many strains of E. coli (see Wilkinson, 1977). The relationship between cell wall composition and inhibition of binding by sugars for C. parvum and different strains of K. aerogenes and S. typhimurium is dealt with separately below.

1.5 Inhibition of binding of C. parvum to macrophages by soluble antigen and extracts of C. parvum.

In an attempt to elucidate which cell wall components were involved in the binding of C. parvum to macrophages, inhibition experiments were performed using culture supernatants and acid and lipid extracts of C. parvum. It can be seen from Table 7

Table 7:

Effects of derivatives of C. parvum on the binding of C. parvum organisms to mouse peritoneal macrophages.

Macrophage monolayers pre-incubated with:	Macrophages binding <u>C. parvum</u> , percentage of untreated control. SE	
<u>C. parvum</u> (10390) culture supernatant	48.2	5.5
Dialyzed <u>C. parvum</u> -supernatant	29.9	4.3
Dialyzed <u>C. parvum</u> -supernatant with 2 mM Ca ⁺⁺ and 2 mM Mg ⁺⁺ added	42.1	5.5
Dialyzed acid extract of <u>C. parvum</u>	82.9	7.3
Dialyzed acid extract of <u>C. parvum</u> with 2 mM Ca ⁺⁺ and 2 mM Mg ⁺⁺ added	36.6	4.9
Dialyzed lipid extract of <u>C. parvum</u>	32.3	4.9
Dialyzed lipid extract of <u>C. parvum</u> with 2 mM Ca ⁺⁺ and 2 mM Mg ⁺⁺ added	62.8	6.7
Dulbecco's PBS + B	100.0	7.9

Pooled data from two separate experiments, each performed in duplicates.
SE: standard error of the percentage converted into percentage of control binding value (see Material and Methods).

that preincubation of the macrophages with each of these derivatives of C. parvum could inhibit its binding. The dialyzed acid extract was not inhibitory until Ca^{++} - and Mg^{++} -ions had been added and this was in accordance with a requirement for these ions for the binding of C. parvum organisms to macrophages (see below). Additions of these ions diminished the inhibitory action of the lipid extract but had no effect on the inhibitory capacity of the culture supernatant.

Both the culture supernatant and the acid extract reacted at a titre of 1/64-1/128 with rabbit anti-C. parvum antiserum in counterimmunoelectroosmophoresis. The lipid extract showed no reaction with the antiserum. According to Dawes et al. (1974) HCl hydrolysis of C. parvum yields a soluble antigen identical to that found in the culture supernatants of the organism.

The dialyzed culture supernatant was as effective in inhibiting binding as the undialyzed supernatant. It was, therefore, unlikely that the binding inhibition by the supernatant was caused by the presence of residual carbohydrates in the bacterial growth medium.

The ability of both acid and lipid extracts to inhibit the binding of C. parvum organisms suggests that both carbohydrate and lipid cell wall components are involved in the binding of the organisms. The similarity in requirement for divalent cations for inhibition by the acid extract and binding of the

whole organisms might, however, indicate that cell wall polysaccharides play a more important part than lipid components. The culture supernatant probably contained sufficient divalent cations from the bacterial growth medium, possibly complexed with larger molecules as dialysis had no effect on its inhibitory activity.

1.6 Inhibition of binding of *C. parvum* to macrophages by sugars and sugar derivatives.

The effect of preincubation of macrophage monolayers with several different sugars and sugar derivatives on subsequent binding of *C. parvum* was tested. The results are shown on Figure 12. It was clear that nine of the sugars tested were potent inhibitors of binding; these were glucose, galactose, fructose, fucose, rhamnose, glucuronic acid, glucosamine, galactosamine and N-acetylglucosamine. One hexose, mannose, the two pentoses xylose and arabinose, N-acetylneuraminic acid and all the sugar alcohols tested had no inhibitory effect on binding. Acetate and succinate were also clearly non-inhibitory whilst pyruvate appeared to have a borderline effect. Dawes *et al.* (1974) have analyzed the composition of the soluble antigen derived from *C. parvum*. This consists in large part of an acidic polysaccharide which contains the following sugars: galactose (0.52 $\mu\text{mol/mg}$), glucose (0.32 $\mu\text{mol/mg}$), mannose (trace amounts), fucose (0.23 $\mu\text{mol/mg}$), N-acetylgalactosamine

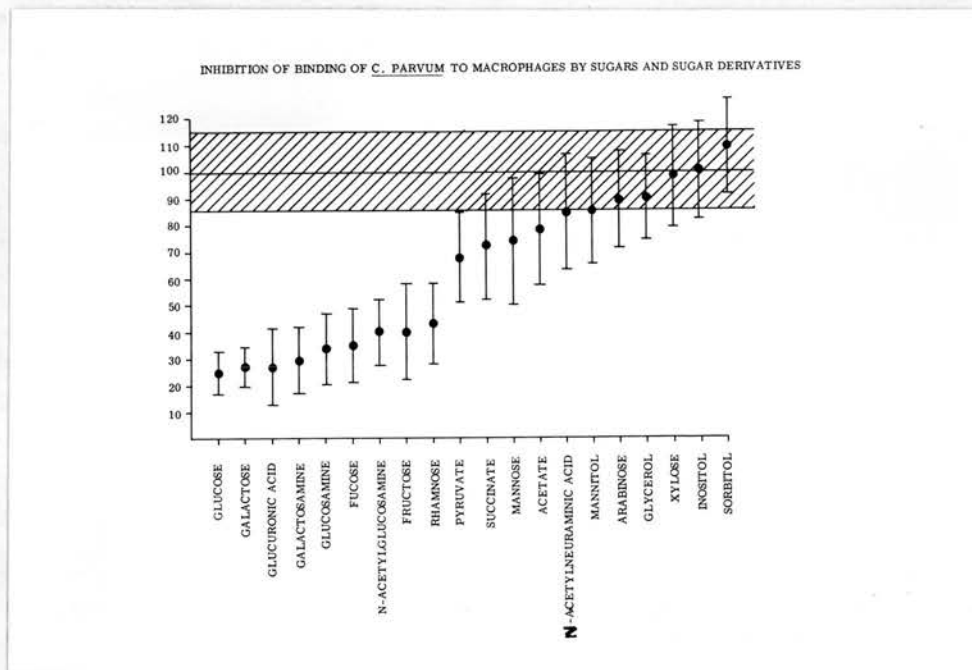


Figure 12. Each point represents the mean of 3-7 replicates, bars indicate 2 standard errors of the percentage (converted into percentage of control binding value, see "Materials and Methods", 14). Binding to untreated controls: 100%, shaded area shows 2 standard errors of the percentage above and below the control value.

(0.19 $\mu\text{mol/mg}$), N-acetylglucosamine (0.43 $\mu\text{mol/mg}$), sialic acids (0.20 $\mu\text{mol/mg}$) and uronic acids (0.23 $\mu\text{mol/mg}$). It can be seen, therefore, that of the nine good inhibitors all apart from fructose and rhamnose are components of the cell wall antigen of *C. parvum* (glucosamine and galactosamine in the N-acetylated

form). All the known cell wall components tested were good inhibitors with the exception of mannose and N-acetylneuraminic acid.

From radioimmunoassay inhibition experiments performed by Dawes & McBride (1975) it appears that N-acetylglucosamine is a major antigenic determinant of the soluble C. parvum antigen. In their experiments antigen-antibody binding was also strongly inhibited by acetate and pyruvate.

1.7 Inhibition of binding of different strains of Klebsiella aerogenes to macrophages by sugars.

Binding inhibition experiments were performed with three different strains of Klebsiella aerogenes; the wild type (NCTC 5055) and two non-capsulated mutants M 2 and M 10B. The mutant M 10B also has a defective lipopolysaccharide core. These strains were tested with macrophages that had been pre-incubated with 5 different sugars that were known to be either present on or absent from the different bacterial strains. It can be seen from Table 8 that binding of the capsulated wild type (5055) was inhibited only by glucose. Binding of the non-capsulated mutant M 2 was inhibited by galactose, glucose and glucosamine whilst only glucose and glucosamine inhibited the binding of the core mutant M 10B. Mannose and arabinose did not inhibit binding of any of the strains. The lipopolysaccharide of the non-capsulated core mutant M 10B is deficient in galactose,

Table 8:

Inhibition of binding of three strains of *K.aerogenes* to mouse peritoneal macrophages by monosaccharides.

<i>Klebsiella aerogenes</i> :	Binding (expressed as percentage of control value) to macrophages pre-incubated with:					
	Galactose (SE)	Glucose (SE)	Mannose (SE)	Glucosamine (SE)	Arabinose (SE)	Dulbecco's PBS + B (SE)
Wild type 5055	92.3 (5.9)	55.2 (5.9)	94.5 (7.3)	89.5 (8.3)	116.6 (8.0)	100.0 (3.3)
Non-capsulated mutant M 2	46.8 (4.7)	58.4 (5.8)	88.4 (6.9)	58.4 (5.8)	114.2 (8.8)	100.0 (3.1)
Core mutant M 10B	98.9 (5.8)	59.2 (5.8)	95.3 (7.9)	52.4 (4.7)	101.0 (7.3)	100.0 (2.8)

Pooled data from two to three separate experiments each performed in duplicates. (SE): Standard error of the percentage converted into percentage of control binding value.

containing only 0.2% (as percentage of dry weight lipopolysaccharide) compared with between 20 and 30% in the wild type and the mutant M 2 (Foxton & Sutherland, 1976). All the strains contain about 5-7% glucose in their lipopolysaccharides and glucosamine is also present in all three strains. The ability of glucose, galactose and glucosamine to inhibit binding of the two mutant strains, therefore, correlates well with the presence or absence of these sugars in the bacterial cell wall. The capsule of the wild type contains both glucose and mannose but no galactose. As the capsule covers the cell wall sugars galactose would not be expected to take part in binding and this explains why this sugar does

not inhibit the binding of the wild type organism despite being a component of the lipopolysaccharide. From the present data the mannose in the capsule does not appear to be involved in binding. This sugar is linked at a branch point to glucuronic acid and might therefore not be expressed in a manner suitable for binding reactions. The presence of large amounts of non-acetylated galactose in the wild type and the non-capsulated mutant M 2 (Poxton & Sutherland, 1976) suggests that the O antigen-specific side chain of the lipopolysaccharide is of type 1 which does not contain mannose (Björndal et al., 1971). Arabinose is also not a known component of any of the three Klebsiella-strains which is in accordance with its inability to inhibit binding.

1.8 Inhibition of binding of different strains of Salmonella typhimurium to macrophages by sugars.

The binding of four different strains of Salmonella typhimurium with a well defined lipopolysaccharide structure to macrophage monolayers was studied in a series of inhibition experiments performed as already described. The results are shown on Table 9. The binding of the wild type (SL 1542) was inhibited consistently by glucose, galactose, glucosamine and rhamnose. Mannose, keto-deoxyoctonate and arabinose had either a borderline or no inhibitory effect. Lipid A was clearly

Table 9:

Inhibition of binding of four strains of <i>S. typhimurium</i> to mouse peritoneal macrophages by monosaccharides									
<i>Salmonella typhimurium</i>	Binding (expressed as percentage of control value) to macrophages pre-incubated with:								
	Glucose (SE)	Galactose (SE)	Glucosamine (SE)	Rhamnose (SE)	Mannose (SE)	KDO (SE)	Lipid A (SE)	Arabinose (SE)	Dulbecco's PBS + B (SE)
SL 1542	51.6 (4.8)	52.8 (4.8)	51.6 (4.8)	40.3 (4.3)	76.6 (6.5)	69.8* (9.0)	32.8** &(6.1) 81.7 (5.6)	94.8 (7.0)	100.0 (2.6)
SL 1096	51.5 (4.6)	54.2 (5.3)	101.1 (5.7)	55.7 (3.8)	81.3 (5.3)	not done	45.3 (4.4)	93.9 (5.7)	100.0 (2.3)
SL 1099	50.8 (4.9)	90.7 (6.1)	96.7 (6.1)	75.6 (5.7)	74.4 (5.3)	not done	19.9 (3.1)	103.3 (6.1)	100.0 (2.4)
SL 1102	98.9 (6.0)	101.9 (6.7)	86.5 (6.0)	82.4 (6.0)	89.5 (6.0)	45.5* (5.8)	14.9 (2.6)	97.0 (6.0)	100.0 (2.4)

Pooled data from two to three separate experiments each performed in duplicates. (SE): Standard error of the percentage converted into percentage of control binding value. *: Only one experiment, performed in duplicates, SE for controls: 9.5 for SL 1542 and 8.0 for SL 8.0. **: Four experiments were performed, each of the figures is the average for two experiments. The standard error for the controls was 6.0 and 5.1 in the first two and second two experiments respectively. KDO: keto-deoxyoctonate.

inhibitory in the first two experiments but not in the following two. As the inhibitory effect of lipid A on the binding of the other strains of *S. typhimurium* tested was also somewhat less marked in later experiments compared with earlier ones it is possible that storage in solution was affecting the material. Binding of SL 1096 was clearly inhibited by glucose, galactose, rhamnose and lipid A. Binding of SL 1099 was markedly inhibited only by glucose and lipid A and only keto-deoxyoctonate and lipid A could inhibit the binding of SL 1102.

Table 10 shows the pattern of binding inhibition by

Table 10:

Inhibition of Binding of Smooth and Rough Strains of *S. Typhimurium* to Macrophages by Monosaccharides and Lipid A.

	O SOMATIC REPEAT UNIT	OUTER CORE	INNER CORE	
	$\left[\begin{array}{c} \text{O Acetyl - Abe} \quad \text{Glc} \\ \quad \\ 1 \quad 3 \\ \quad \\ \text{Man - Rha - Gal} \end{array} \right]_n$	$\begin{array}{c} \text{GlcNac} \quad \text{Gal II} \\ \quad \\ \text{Glc II - Gal I -} \quad \text{Glc I - Hep II - Hep I} \end{array}$	$\begin{array}{c} \text{KDO} \\ \\ \text{Lipid A} \\ \\ \text{Ethanolamine} \end{array}$	
Genotype	+	rfaK	rfaE	
Chemotype	smooth	Rb	Re	
STRAIN	SL 1542	SL 1096	SL 1099	SL 1102
INHIBITION BY:				
GLUCOSE	+	+	+	-
GALACTOSE	+	+	-	-
GLUCOSAMINE	+	-	-	-
RHAMNOSE	+	+	-	-
MANNNOSE	-	-	-	-
KDO	ND	ND	ND	+
LIPID A	+ -	+	+	+
ARABINOSE	-	-	-	-

+ inhibition, - no inhibition

Abbreviations Glc = glucose, Gal = galactose, Abe = abequose, GlcNac = N-acetyl glucosamine, Man = mannose, Rha = rhamnose, Hep = L-glycero-D-mannoheptose, KDO = 2-keto-3-deoxyoctonate, ND = not done.

monosaccharides and lipid A in relation to the lipopolysaccharide structure of the 4 strains of *S. typhimurium* (see Lüderitz *et al.* 1974). It can be seen that there was in most cases good correlation between the ability of a sugar to inhibit binding of organisms of a particular strain and its presence in the cell wall of that strain. Mannose would have been expected to inhibit binding of SL 1542 but this sugar is linked at a branch point to abequose (see Table 10) which may prevent it from taking part in binding reactions in a similar way as was suggested above for the mannose in the capsule of *K. aerogenes*

NCTC 5055. The mutant SL 1096 should not express rhamnose and yet its binding was inhibited by this sugar. This may be explained by the possibility that this mutant is "leaky" and synthesising and expressing very small amounts of the wild type polysaccharide (Lehmann et al., 1973). Binding of the two most deficient strains, SL 1099 and SL 1102 is inhibited only by the few sugars left in their short lipopolysaccharides with lipid A clearly playing a more important role in binding as less is left of the polysaccharide chain (see Table 9). The ability of lipid A to inhibit binding of strains with more complex polysaccharide chains may possibly be explained by its galactose content (see Lüderitz et al., 1973) as it would not be expected to be very freely expressed in these strains. Keto-deoxyoctonate does not contribute significantly to the binding of SL 1542 where a complex polysaccharide chain is linked to this sugar whilst the binding of SL 1102 with freely expressed keto-deoxyoctonate is inhibited by this sugar.

2. THE ROLE OF DIVALENT CATIONS IN THE BINDING OF NON-OPSONIZED BACTERIA TO MACROPHAGES

2.1 The role of Ca^{++} - and Mg^{++} -ions in the binding of

C. parvum to macrophages.

The requirement for Ca^{++} - and Mg^{++} -ions in the binding of *C. parvum* to macrophage monolayers was studied in a series of experiments where free Ca^{++} - and Mg^{++} -ions were removed from the macrophage monolayers and bacterial suspension with EDTA

and washings and the binding assay then performed in the presence of known concentrations of Ca^{++} and Mg^{++} (see "Materials and Methods", 9.3.). Figures 13 and 14 show the effect of removing divalent cations and adding different known concentrations of Ca^{++} and Mg^{++} on the binding of C. parvum at 4°C and at room temperature (20°C). It can be seen that binding did not occur at either temperature in the complete absence of divalent cations. Both Ca^{++} and Mg^{++} were required for binding at 4°C whilst at room temperature some binding was seen in the presence of Mg^{++} alone. It had also been noted in preliminary experiments that Mg^{++} -ions alone could promote binding and phagocytosis of C. parvum at 37°C almost as effectively as Ca^{++} and Mg^{++} together whilst Ca^{++} -ions alone were less effective.

Dulbecco's PBS + B, the medium normally used in the binding assays, contains 1 mM Ca^{++} and 0.5 mM Mg^{++} .

3. THE EFFECT OF ENZYMES AND CHEMICAL TREATMENT ON THE BINDING OF UNOPSONIZED BACTERIA TO MACROPHAGES

3.1 The effect of treatment of macrophages with various enzymes on the binding of C. parvum and recovery from the effect.

In order to investigate the nature of the cell surface components of macrophages involved in the binding of C. parvum macrophage monolayers were exposed to various enzymes and the binding of C. parvum tested either directly following this

THE EFFECT OF DIVALENT CATIONS ON THE
BINDING OF *C. PARVUM* TO MACROPHAGES
AT 4°C. ●● : Ca^{++} ■■ : Mg^{++} ▲▲ : $\text{Ca}^{++} + \text{Mg}^{++}$

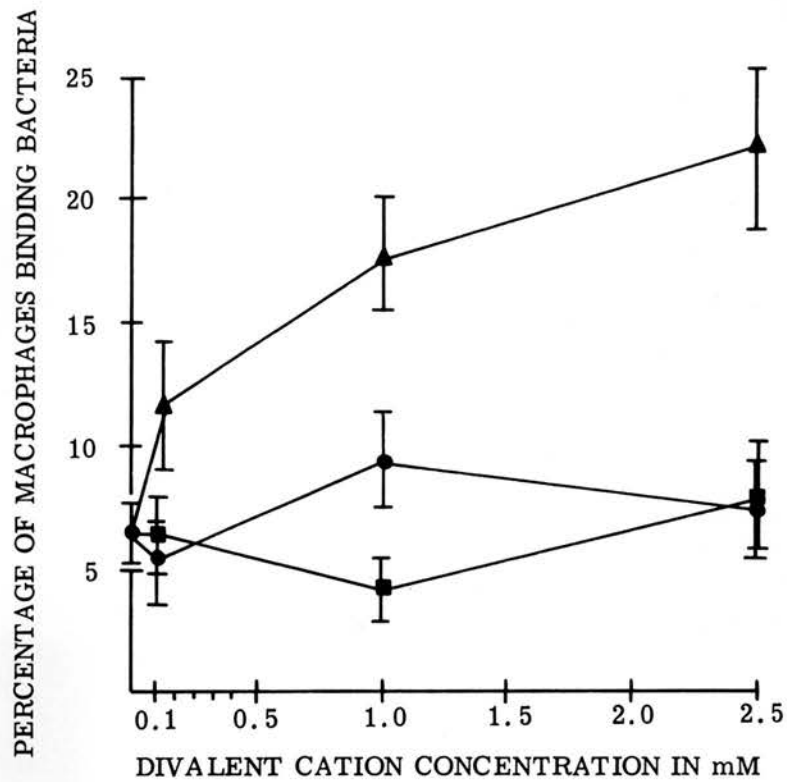


Figure 13. Each point represents the mean of 3-6 replicates.
Bars indicate 2 standard errors of the percentage.
Pooled data from 2 representative experiments.

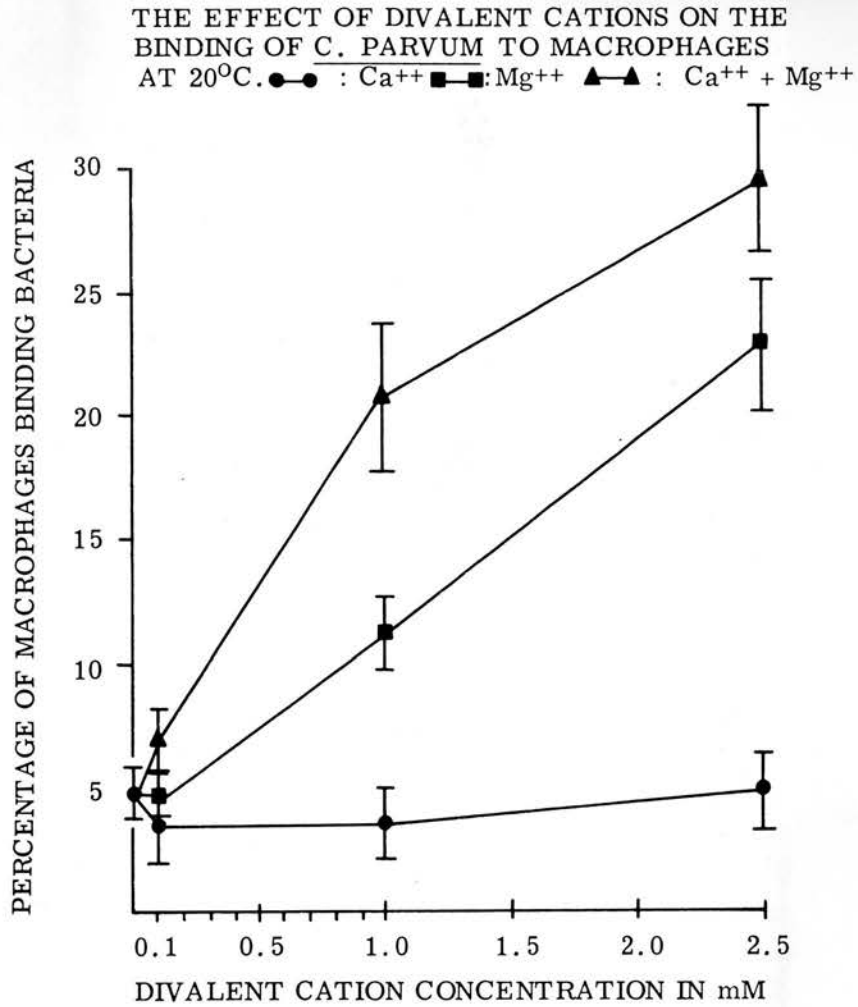


Figure 14. Each point represents the mean of 3-6 replicates.
Bars indicate 2 standard errors of the percentage.
Pooled data from 2 representative experiments.

treatment or after the cells had been incubated for one hour at 37°C in tissue culture medium (199 or Eagle's MEM). The results are summarized in Table 11. It can be seen that all the enzymes used apart from neuraminidase caused a decrease in binding to 40-60% of the binding to untreated controls. Treatment with neuraminidase led to a 30% increase in binding. Higher concentrations did not have a much greater effect unless they

Table 11:

The effect of enzymes on binding of *C. parvum* to PE cell monolayers, recovery during 1 h in tissue culture medium and the effect of EDTA on recovery

PE cell monolayers preincubated at 37°C for 12 min with	PE cells binding <i>C. parvum</i> , percentage of control					
	Direct assay		Recovery incubation, 1 h at 37° with culture medium containing			
		s.e.	No EDTA		10 mM EDTA	
Trypsin 1 mg/ml†	47.7	3.0	96.8	6.4	43.8	4.6
Pronase 250 µg/ml†	47.7	3.3	35.7	3.3	43.9	3.7
β-galactosidase 0.05 e.u./ml‡	53.9	3.1	87.2	6.4	62.1	5.5
Neuraminidase 5 u/ml†	131.9	4.8	91.5	5.6	100.9	6.4
Phospholipase A 1.0 u/ml§	46.3*	4.2	91.1*	7.4	81.1*	6.8
Phospholipase C 20 µg/ml†	42.6	2.6	91.3	5.0	81.3	5.0
Phospholipase D 0.05 e.u./ml¶	47.0	3.4	91.3	6.4	73.5	8.2
Dulbecco's PBS + B + glycerol 2.7%	78.5	4.5	78.7	6.2	81.0	6.2
Dulbecco's PBS + B	100.0		91.5	4.0	95.5	4.0

Each figure is the mean of at least 4 replicates, data from two or more experiments pooled.

* Percentage of control with corresponding concentration of glycerol.

† In Dulbecco's PBS + B, pH 7.2.

‡ In Dulbecco's PBS + B containing 0.05mM Mn²⁺, pH 7.2.

§ Stored in 50% glycerol, diluted in physiological saline containing 10mM Ca²⁺, pH 7.4.

¶ In Dulbecco's PBS + B, pH 5.6. Binding was shown not to be affected by the low pH alone.

PE cell monolayers: peritoneal exudate cell monolayers consisting of approximately 95% macrophages; s.e.: standard error of the percentage converted into percentage of control binding value.

were also toxic for the cells as shown by decreased adherence to the coverslips and decrease in trypan blue exclusion. Thus trypsin at 10 mg/ml reduced binding to 9.6% of control levels but many cells had detached from the coverslips and only 77% of those remaining excluded trypan blue under these conditions. Treatment with phospholipase A at 1.5 U/ml and 2.0 U/ml decreased binding to 45.0% and 19.5% of glycerol-treated controls, respectively. Macrophages that had been exposed to these concentrations of phospholipase A were easily washed off the coverslips and of those that remained attached only 71% excluded trypan blue after exposure to 1.5 U/ml of phospholipase A. With the highest concentration of phospholipase A the cells were clearly disintegrating. Exposure to 40 μ g/ml and 50 μ g/ml of phospholipase C reduced the binding to 33.5% and 18.7% of untreated controls. This was also accompanied by a marked loss of cells from the coverslips and reduction in viability of those remaining to 70% for phospholipase C at 40 μ g/ml. The cells that were washed away from the coverslips can be assumed to have been dead as viable macrophages are not easily detached from a surface. Viability of macrophages on control coverslips was 90%. β -galactosidase and phospholipase D could not be completely dissolved at higher concentrations.

These results would indicate that an intact cell surface is important for the binding of non-opsonized bacteria to macrophages and that many different components contribute about

equally to the integrity of the plasma membrane in this respect. The increase in binding following treatment with neuraminidase was probably related to a reduction in surface negative charge, see "Discussion".

The right hand part of Table 11 shows how the macrophages recover from the consequences of enzyme treatment during one hour in tissue culture medium and the effect of EDTA on the recovery. Before the "recovery" incubation the monolayers were thoroughly washed with Dulbecco's FBS + B in order to remove the enzyme solutions. As shown in Table 11 incubation for one hour at 37°C led to the return of binding ability to control levels in all cases except for pronase where the enzyme effect increased during this period. EDTA had different effects on recovery depending on which enzyme the cells had been exposed to. Recovery from treatment with trypsin and β -galactosidase was completely prevented in the absence of free divalent cations, whereas return to normal following treatment with the other enzymes was only slightly affected (viz. phospholipases A, C and D) or unaffected by EDTA (viz. neuraminidase). Pronase-treated cells showed a higher degree of binding after "recovery" in EDTA-containing medium than medium without EDTA and this was similar to the degree of binding observed directly after exposure to pronase. This was also evident when a higher concentration of pronase (1000

$\mu\text{g/ml}$) was used where the binding was 48.9% of the control value directly after exposure to the enzyme, decreased to 2.1% during one hour in the tissue culture medium but was 32.4% if the medium contained EDTA. It is known that some components of pronase require Ca^{++} for their stability (Awad et al., 1972).

The results show that the surface components of macrophages involved in binding of unopsonized bacteria that are affected by brief exposure to enzymes can return to normal quite rapidly following removal of the enzymes. It is also suggested that divalent cations may be involved in maintaining the integrity of plasma membrane components that mediate binding as well as playing a role in binding itself as implied by the results of the preceding chapter. The repair mechanism for different components seem to vary in their susceptibility to divalent cation depletion.

3.2 The effect of treatment of macrophages with periodate on the binding of *C. parvum* and reversal of the effect by borohydride.

In order to investigate the role of carbohydrates on the macrophage surface in binding of bacteria the cells were exposed to periodate and borohydride. Mild treatment with periodate has been shown to remove the terminal two carbon atoms from sialic acid and oxidize the alcohol group on carbon 7 to aldehyde. Subsequent treatment with borohydride reduces this aldehyde group back to an alcohol group. The rest of the sialic acid molecule

and other carbohydrates are not affected under these mild conditions (Van Lenten & Ashwell, 1971). As shown in Table 12 brief exposure to 1 mM periodate (10^{-4}) at 37°C affected binding of C. parvum to macrophages to a similar degree as was seen after treatment with enzymes (apart from neuraminidase). Treatment with periodate for 10 or 15 minutes at room temperature had a similar effect (38.0% and 48.6% binding as compared with

Table 12:

The effect of periodate on the binding of <u>C. parvum</u> to PE cell monolayers and reversal by borohydride		
PE cell monolayers preincubated with	PE cells binding <u>C. parvum</u> percentage of control	
		s.e.
Dulbecco's PBS + B, 5 min, 37°	100.0	
IO ₄ ⁻ 1mM, 5 min, 37°	39.6	3.5
IO ₄ ⁻ 1mM, 5 min, 37°, followed by Eagle's MEM, 60 min, 37°	45.5	4.5
IO ₄ ⁻ 1mM, 5 min, 37°, followed by BH ₄ ⁻ , 1mM, 5 min, 37°	81.7	5.4
IO ₄ ⁻ 1mM, 5 min, 37°, followed by Dulbecco's PBS, 5 min, 37°	44.6	4.5

Each figure is the mean of 5-7 replicates, pooled data from 3 experiments.

PE cell monolayers: peritoneal exudate cell monolayers consisting of approximately 95% macrophages; s.e.: standard error of the percentage converted into percentage of control binding value.

controls, respectively). Exposure to this concentration of periodate for longer than 5 minutes at 37°C resulted in marked loss of viability of the cells and reduced binding to 3.5% of control values. The shorter treatment had no significant effect on cell viability.

In contrast to the rapid recovery observed following treatment with enzymes incubation of periodate-treated cells with tissue culture medium for one hour at 37°C after the periodate had been washed away did not lead to a return to normal binding ability. The effect of periodate could, however, be largely reversed by subsequent brief exposure to an equal concentration (1 mM) of borohydride (BH_4^-) at 37°C before adding the bacterial suspension.

These data might indicate an involvement of hydroxyl groups on carbohydrates such as sialic acids in the binding of C. parvum to macrophages.

4. MECHANISMS OF MACROPHAGE ACTIVATION

In the second part of this work a possible mechanism for signal transmission following surface binding of an activating agent to macrophages was studied. This chapter describes the effect of various stimuli on the turnover of phosphatidyl-inositol by macrophages as measured by the uptake of labelled myo-inositol. In the final chapter of this section the occurrence and time course of enhanced phosphatidylinositol turnover are related to two effector functions of activated macrophages, bacteriostatic effect against Listeria and production of lysosomal enzymes.

4.1 Uptake of myo-(2-³H)-inositol by macrophages.

In order to investigate the possibility that an increase in turnover of phosphatidylinositol is involved in the mediation of macrophage activation the effect of several different stimuli on the uptake of myo-(2-³H)-inositol by macrophages was tested. As explained in the "Introduction" (13.1.6.) the degree of uptake of myo-(2-³H)-inositol measured over a defined period will reflect the rate of turnover of phosphatidylinositol as apparently only the diglyceride backbone is recycled in the process (see Figure 1 in the "Introduction"). In the present study the uptake of tritiated myo-inositol during one hour was measured as described in "Materials and Methods" (12.1).

When 2 μCi ($= 0.07 \mu\text{g}$) were added to each culture tube the measured uptake was usually in the range of 200-500 counts per minute per 2×10^6 to 3×10^6 macrophages. The culture medium was serum-free Eagle's MEM which contains 2 mg/l of cold myo-inositol (giving 6 μg per culture tube). The added radioactive material was thus 1.2% of the total myo-inositol present in the medium. Increasing the concentration of labelled myo-inositol up to 10 μCi per culture tube or using medium free of cold inositol did not consistently result in higher counts.

The cells took up only approximately 0.01% of the added radioactive material. Most of the radioactivity (50-90%) could be recovered in the culture medium and the remainder in the solution used to wash the cells after the incubation. Less than one percent was found in the aqueous phase from the first equilibration with the lipid extract whilst only background counts were obtained with samples from the second aqueous phase.

The absence of radioactivity from the second aqueous phase shows that water-soluble myo-(2- ^3H)-inositol was effectively eliminated from the lipid extract. Control experiments had also shown that a brief pulse of radioactive myo-inositol followed by immediate lipid extraction of the cells resulted in no uptake into the lipid phase.

Thin layer chromatography of the macrophage lipid extract showed that approximately 95% of the total lipid radioactivity was associated with phosphatidylinositol and most of the remainder

was found in a fraction including phosphatidylethanolamine, phosphatidic acid, cardiolipin and neutral lipids.

4.2 The effect of various stimuli on the turnover of phosphatidylinositol by macrophages.

Macrophage monolayers were exposed to various stimuli for four hours in serum-free medium and the uptake of myo-(2-³H)-inositol during the last one hour measured. The results are summarized in Table 13 which also gives the concentrations of substances and particles used. It can be seen that both endotoxin and C. parvum caused an increase in the uptake of

Table 13:

The effect of various stimuli on the uptake of myo-(2-³H)-inositol by macrophages.

Macrophages exposed for 4 h to:	Uptake of <u>myo</u> -(2- ³ H)-inositol (expressed as percentage of uptake by unstimulated controls)	
	Without T-cells	In presence of T-cells
Endotoxin (E. coli, 055:B5 Difco), 60 µg/10 ⁶ cells	129.1 SE 4.7 p<0.001	129.7 SE 9.5 0.01<p<0.02
<u>Corynebacterium parvum</u> (10390) 100 bact./cell	106.3 SE 5.4 n.s.	122.9 SE 6.5 0.02<p<0.025
Latex particles (0.81 µm, Difco) 500 particles/cell	93.9 SE 4.9 n.s.	106.7 SE 10.3 n.s.
Carbon particles O.D. 0.36	94.1 SE 2.1 n.s.	96.7 SE 5.3 n.s.
<u>Staph. albus</u> 500 bact./cell	96.6 SE 4.0 n.s.	95.5 SE 9.5 n.s.
Eagle's medium without serum	100.0 SE 4.4-6.0	102.1 SE 2.5-6.5 n.s.

The figures represent means of pooled data from 2-3 experiments, each done in triplicate. Significance of differences was determined using Student's t-test (two-tailed). SE: Standard error of the mean. p values <0.05 are considered significant, n.s. = not significant.

labelled inositol indicating an increased rate of turnover of phosphatidylinositol. Endotoxin had a stimulatory effect both with and without splenic T-cells (prepared as described in "Materials and Methods", 11.3) whilst C. parvum was effective only in the presence of T-cells. The ratio of T-cells to macrophages was 1:5. The T-cells alone had no effect on the uptake of tritiated myo-inositol. On microscopical examination of parallel cultures on coverslips or in microtitre wells some of the lymphocytes were observed to cluster around the macrophages. The lymphocytes were seen to be completely removed by the washing procedure at the end of the experiment and did therefore not contribute to the measured radioactivity.

The results indicate a stimulation of turnover of macrophage phosphatidylinositol by the macrophage activating agents endotoxin and C. parvum whilst the other particles do not have this effect. In contrast to endotoxin C. parvum is stimulatory only in the presence of T-cells.

4.3 Phagocytosis of particles and adherence of macrophages during assay for phosphatidylinositol turnover.

The degree of phagocytosis of particles that were tested for their effect on phosphatidylinositol turnover was determined in parallel cultures on coverslips. The coverslips were heat-fixed and Gram-stained except for those containing latex particles which were left unfixed, inverted wet on to a microscopic slide

and examined immediately using phase contrast optics. During 4 hours in serum-free medium C. parvum was taken up by 33% of the adherent cells, latex particles by 45%, carbon particles by 16% and St. albus by 23%. When splenic T-cells were added to the macrophage monolayers at a ratio of 1:5 at the beginning of the culture period, but washed away before fixation, the uptake of C. parvum by the macrophages was unchanged (35%). These data imply that the observed increases in turnover of phosphatidylinositol following exposure of macrophages to endotoxin or C. parvum with T-cells but not the other test particles is not directly related to the process of phagocytosis.

The concentrations of endotoxin and C. parvum used in the assay for myo-(2-³H)-inositol uptake did not affect the viability of parallel cultures of macrophages on coverslips. Viability (by trypan blue exclusion) of endotoxin-treated cells was 89% (controls 92%) and C. parvum-treated cells 94% (controls 97%).

The tube cultures were usually checked at the end of the culture period under an inverted microscope for changes in morphology and adherence to the glass. After 4 hours no morphological differences were evident between treated cells and controls. By using an ocular grid the relative numbers of adherent cells could be estimated. No apparent differences in numbers of adherent cells between test cultures and control cultures could be detected. Two examples are given in Table 14.

Table 14:

Adherence of mouse peritoneal macrophages following 4 hours of culture with bacterial endotoxin or C. parvum.

	Macrophage monolayers cultured with:	
	Eagle's MEM	Endotoxin
Average number of adherent cells per 6 squares counted	83	82

One representative experiment

	Macrophage monolayers cultured with:			
	Eagle's MEM	T-cells	<u>C. parvum</u>	<u>C. parvum</u> + T-cells
Average number of adherent cells per 4 squares counted	44	46	43	43

One representative experiment.

Coverslip cultures of macrophages exposed to endotoxin or C. parvum yielded similar results; the total number of adherent cells per coverslip was 8.8×10^5 in endotoxin-treated cultures compared with controls of 9.8×10^5 , cultures exposed to C. parvum had 1.9×10^5 cells per coverslip compared with 1.7×10^5 on control coverslips. The differences observed in uptake of labelled inositol are thus not likely to have been caused by changes in the number of adherent cells.

4.4 Effects of different concentrations of C. parvum and different incubation periods on phosphatidylinositol turnover by macrophages.

In an attempt to achieve stimulation of uptake of labelled

myo-inositol by macrophages with C. parvum in the absence of T-cells different concentrations of C. parvum were employed and the effect of prolonged incubation was also tested.

Concentrations of C. parvum higher than 10^8 bact./ 10^6 macrophages were found to be toxic and resulted in reduced viability as tested by trypan blue exclusion and judged by the loss of adherent cells with a corresponding decrease in uptake of myo-(2- 3 H)-inositol (about 15% decrease for 3×10^{10} bact./ 10^6 cells).

In preliminary experiments several concentrations of C. parvum including those that were non-toxic had been added with labelled myo-inositol to macrophage monolayers for a total incubation period of one hour. This never resulted in stimulation of uptake; non-toxic doses of C. parvum had no effect on the uptake which was 94-96% of that of untreated controls. It is, therefore, unlikely that a rapidly occurring response was being missed.

Table 15 shows that prolonged incubation with C. parvum for up to 4 days led to decrease rather than increase in the uptake of labelled myo-inositol. This was not correlated with an obvious decrease in cell numbers as compared with controls; both control and treated cultures showed a decline in cell numbers of about 12-20% between the second and fourth day of culture.

These results indicate that there was an absolute requirement

Table 15:

Effect of prolonged culture with C. parvum on the uptake of myo-(2-³H)-inositol by mouse peritoneal macrophages.

Macrophages exposed to <u>C. parvum</u> (100 bact. per cell) for:	Uptake of <u>myo</u> -(2- ³ H)-inositol (expressed as percentage of uptake by unstimulated controls).		
24 hours*	92.1	SE: 13.0	n.s.
48 hours	64.1	SE: 3.4	p<0.001
72 hours	87.2	SE: 10.9	n.s.
96 hours	64.3	SE: 12.3	0.025<p<0.05

*: One experiment only, performed in triplicates. Other values are based on pooled data from three experiments, each performed in triplicates, except two experiments for 96 hours. SE: Standard error of the mean; SE for controls were 5.1, 5.9, 6.0 and 10.2 for 24, 48, 72 and 96 hours respectively. Significance of differences determined by two-tailed Student's t-test, n.s.: not significant.

for T-cells for the stimulation of phosphatidylinositol turnover by C. parvum.

4.5 Requirement for T-cells for stimulation of phosphatidylinositol turnover by macrophages with C. parvum.

The requirement for T-cells for stimulation of phosphatidylinositol turnover by macrophages with C. parvum was studied further. Table 16 shows that whole spleen cells were apparently as effective as enriched T-cells in promoting stimulation with C. parvum and that, therefore, the effect of the enriched T-cells was not an artefact of the nylon-wool filtration procedure. It will be noted, however, that in these experiments C. parvum caused a small but significant increase in uptake of myo-inositol

Table 16:

The effect of the presence of whole spleen cells on the stimulation by C. parvum of phosphatidylinositol turnover by macrophages.

Macrophages exposed for 4 hours to:	Uptake of myo-(2- ³ H)-inositol (expressed as percentage of uptake by unstimulated controls).		
Whole spleen cells (2.5 spleen cells/ macrophage)	115.8	SE: 2.4	p<0.001
<u>C. parvum</u> (100 bact./macrophage)	113.1	SE: 3.9	0.02<p<0.025
<u>C. parvum</u> and whole spleen cells	130.1	SE: 3.1	p<0.001
Eagle's medium without serum	100.0	SE: 2.8	

Pooled data from two experiments, each performed in triplicates.

SE: standard error of the mean. Significance of differences determined by two-tailed Student's t-test.

in the absence of lymphocytes. The spleen cells also had a small stimulatory effect when added without C. parvum. At the time when these experiments were performed the mice were affected by an unidentified infection. The morphology of the macrophages resembled that of stimulated or activated macrophages before any treatment and they were shown to be cytotoxic to tumour cells without further stimulation (Gwen Barrow, personal communication). It was mentioned in the "Introduction" (12.1.2; 12.2.3) that macrophages that have already been stimulated in vivo respond more easily and vigorously than normal macrophages to further stimulation in vitro.

The same type of experiment was also performed using enriched T-cells prepared from the spleens of mice immunized

14 days previously with C. parvum. These mice had marked splenomegaly and serum antibodies against C. parvum (latex agglutination test). It can be seen from Table 17 that these T-cells were no more effective than normal T-cells in eliciting the stimulatory effect of C. parvum on phosphatidylinositol turnover by macrophages.

Table 17:

The effect of the presence of immune T cells on the stimulation by <u>C. parvum</u> of phosphatidylinositol turnover by macrophages.			
Macrophages exposed for 4 h to:	Uptake of myo-(2- ³ H)-inositol (expressed as percentage of uptake by unstimulated controls)		
<u>Corynebacterium parvum</u> (10390) 100 bact./cell	110.9	SE 6.3	n.s.
<u>Corynebacterium parvum</u> and immune T cells	118.9	SE 6.2	0.02<p<0.025
Immune T cells	102.2	SE 4.2	n.s.
Eagle's medium without serum	100.0	SE 4.3	

The figures represent means of pooled data from 4 experiments, each done in triplicate. SE: Standard error of the mean. p values determined using two-tailed Student's t-test, p<0.05 significant, n.s. = not significant.

4.6 Stimulation of DNA-synthesis by normal and immune enriched T-cells with *C. parvum*.

As normal and *C. parvum*-immune T-cells were equally effective in inducing stimulation by *C. parvum* of phosphatidylinositol turnover in macrophages it was of interest to study whether both normal and immune T-cells could respond to *C. parvum*. The effect of *C. parvum* on the rate of DNA-synthesis by normal and immune T-cells was therefore tested by measuring the uptake of tritiated thymidine by T-cells cultured in the presence of a small proportion of macrophages and exposed to two different doses of *C. parvum* (see "Materials and Methods", 11.4).

Table 18 shows that the uptake in the absence of further stimulation in vitro by the immune T-cells was higher as compared with normal T-cells but both normal and immune T-cells showed a similar increase in uptake in response to *C. parvum*. The higher ratio of bacteria to cells was equivalent to that used in the assay for phosphatidylinositol turnover. These results were obtained with cultures incubated for 3 days with *C. parvum*. If incubation was prolonged to 6 days the results were rather inconsistent, but usually no significant increase in uptake of (6-³H)-thymidine was observed in response to *C. parvum* by either normal or immune T-cells. The response of the T-cells was

Table 18:

The effect of C. parvum on the uptake of (6-³H)-thymidine by T cells from normal mice and mice immunized 14 days previously with C. parvum.

<u>Corynebacterium parvum</u>	Uptake of (6- ³ H)-thymidine, counts per minute			
	Normal T cells		Immune T cells	
None	450.8	SE 129.4	1008.0	SE 72.4
100 bact. /cell	1372.7	SE 141.3 p<0.001	1251.4	SE 53.4 0.01<p<0.02
500 bact. /cell	1743.0	SE 328.0 0.005<p<0.01	2826.7	SE 310.5 p<0.001

Pooled data from 3-4 experiments, each performed in triplicates.

SE: standard error of the mean. p values determined using two-tailed

Student's t-test, p<0.05 significant, n.s. = not significant.

absolutely dependent on the presence of the low proportion of macrophages (5%). In the absence of macrophages both normal and immune T-cells gave 300-500 counts per minute (c.p.m.) regardless of whether C. parvum had been added or not. Increasing the proportion of macrophages to 10% depressed the response.

Microscopical examination of smears prepared from T-cells and C. parvum mixed at the ratios used in these experiments and incubated for 4 hours showed no obvious direct binding of C. parvum to the T-cells.

In one experiment culturing normal, enriched T-cells with E. coli endotoxin (0.24 µg and 1.20 µg per well) had no effect

on their rate of thymidine uptake; the c.p.m. were 227.4 and 215.5 respectively, with controls giving 189.2 c.p.m.

These results thus demonstrated a macrophage-dependent increase in DNA-synthesis in both normal and immune T-cells in response to C. parvum but no effect of endotoxin on normal T-cells.

4.7 The role of soluble factors derived from lymphocytes in the stimulation by C. parvum of phosphatidylinositol turnover by macrophages.

In order to investigate the possible role of soluble mediators produced by lymphocytes in the stimulation of phosphatidylinositol turnover by macrophages a series of experiments was performed in which the macrophage monolayers were incubated with supernatants from lymphocyte cultures in the presence or absence of C. parvum and the effect on the rate of uptake of labelled myo-inositol tested.

Table 19 shows results obtained with supernatants from normal or immune unfractionated spleen cells cultured under the conditions used in the assay for phosphatidylinositol turnover (4 hours in Eagle's MEM without serum) with or without C. parvum at a ratio of 10^8 bact./ 10^6 cells. It can be seen that supernatants from either normal or immune spleen cells had no stimulatory effect on the uptake of labelled myo-inositol

Table 19:

The effect of supernatants from normal and immune spleen cells cultured with and without C. parvum on the rate of phosphatidylinositol turnover by macrophages in the presence and absence of C. parvum.

	Uptake of myo-(2- ³ H)-inositol (expressed as percentage of uptake by unstimulated controls)			
	Without <u>C. parvum</u>		In presence of <u>C. parvum</u>	
Supernatant from normal spleen cells	90.8	SE 4.7 n.s.	98.9	SE 3.7 n.s.
Supernatant from normal spleen cells cultured for 4 h with <u>C. parvum</u>	109.2	SE 8.7 n.s.	112.0	SE 4.8 n.s.
Supernatant from immune spleen cells	102.4	SE 8.5 n.s.	92.1	SE 6.4 n.s.
Supernatant from immune spleen cells cultured for 4 h with <u>C. parvum</u>	104.9	SE 9.0 n.s.	91.0	SE 8.9 n.s.
Eagle's medium without serum	100.0	SE 3.4-3.9	110.9	SE 5.8-11.6 n.s.

The figures are the means of pooled data from 2-4 experiments, each done in triplicate. SE: Standard error of the mean, p values determined using two-tailed Student's t-test, p<0.05 significant, n.s. = not significant.

by macrophages regardless of whether the spleen cells had been incubated with or without C. parvum. None of these supernatants could induce stimulation of phosphatidylinositol turnover by macrophages with C. parvum (right hand column of Table 19). Supernatants from lymphocyte cultures could thus not mimic the effect of C. parvum added with T-cells (or whole spleen cells) or replace T-cells in the stimulation of macrophages by C. parvum.

In the course of these experiments it was noted that the activity of the supernatants could change on storage and if they were kept for over about 40 hours, particularly if they had

been frozen, they tended to have a marked stimulatory effect even though aliquots of the same supernatants had not shown any stimulatory activity if used after overnight storage. Supernatants kept overnight at 0°C gave reproducible results.

Attempts to stimulate macrophage phosphatidylinositol turnover with lymphokine-containing supernatants from 1 or 3 day mixed lymphocyte cultures using spleen cells from C3H/Buf and CFE mice yielded unsatisfactory results as these supernatants as well as supernatants from unmixed control cells of C3H/Buf or CFE spleen cells were all highly stimulatory. Supernatants from 24hour cultures of syngeneic fibrosarcoma cells or embryonic fibroblasts were also shown to have a stimulatory effect. Cell supernatants can, therefore, have a very non-specific effect on the uptake of labelled myo-inositol by macrophages which may reflect a sensitivity of the macrophages and/or the assay system to changes in the microenvironment. The difficulties in storing cellular supernatants may have contributed to these results.

The viability of the spleen cell cultures declined fairly rapidly with time. In cultures kept for 4 hours approximately 75% of the cells were viable; C. parvum had no effect on the viability but the addition of 5% foetal calf serum increased it to 85%. After 18 hours in the presence of 10% foetal calf serum the viability had fallen to 60%. It is thus possible that cellular breakdown products may have influenced

the results obtained in the phosphatidylinositol turnover assay.

5. EFFECTOR FUNCTIONS OF MACROPHAGES IN RELATION TO INCREASED TURNOVER OF PHOSPHATIDYLINOSITOL.

5.1 Time course of stimulation of phosphatidylinositol turnover and development of increased antibacterial activity by macrophages treated with endotoxin.

The time course of stimulation of phosphatidylinositol turnover by macrophages was studied by measuring the uptake of myo-(2-³H)-inositol after 1,4,6 and 24 hours of incubation with endotoxin. The labelled myo-inositol was always added 1 hour before the end of the culture period.

As can be seen from Figure 15 there was a small, but not significant, increase in uptake after 1 hour ($0.05 < p < 0.10$; SE for control 4.2). The turnover was significantly increased after 4 hours (see Table 13) and was still rising at 6 hours ($0.001 < p < 0.05$; SE for controls 5.8). At 24 hours the uptake had declined to about the same value as at 4 hours but was still significantly raised as compared with controls ($0.005 < p < 0.01$; SE for controls 6.6). Figure 15 also shows that the antibacterial activity of macrophages exposed to endotoxin developed more slowly than the response in phosphatidylinositol turnover.

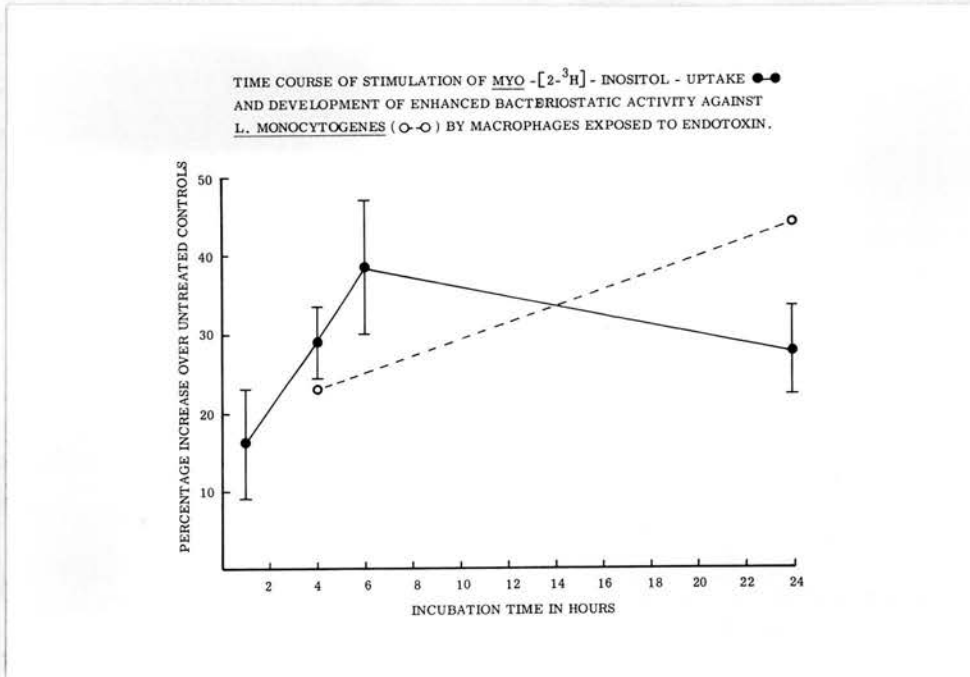


Figure 15. Data on myo-(2-³H)-inositol-uptake from 3-4 experiments, each performed in triplicates. Bars indicate 1 standard error of the mean. Data on bacteriostatic activity from one representative experiment, depicted in detail in Figure 17.

The details of two separate experiments testing the antibacterial activity of macrophages against L. monocytogenes are given in Figures 16 and 17. There was some cell death following internalization of L. monocytogenes; this was less marked in cells that had been treated with endotoxin. The

THE EFFECT OF LIPOPOLYSACCHARIDE (LPS) ON THE BACTERIOSTATIC EFFECT
OF MACROPHAGES AGAINST *LISTERIA MONOCYTOGENES*. (EXPERIMENT 1)

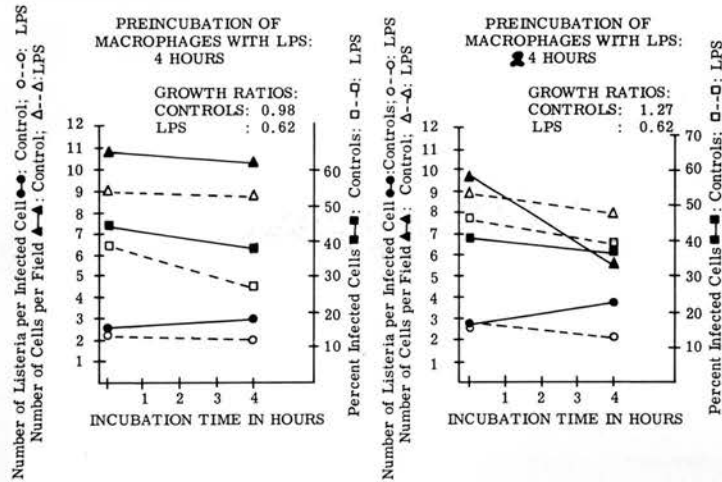


Figure 16. In this experiment penicillin and streptomycin were added after phagocytosis had been allowed to take place and excess *Listeria* washed away; final concentration of each: $5 \mu\text{g/ml} = 1/10$ of the normal tissue culture concentration.

THE EFFECT OF LIPOPOLYSACCHARIDE (LPS) ON THE BACTERIOSTATIC EFFECT
OF MACROPHAGES AGAINST *LISTERIA MONOCYTOGENES* (EXPERIMENT 2)

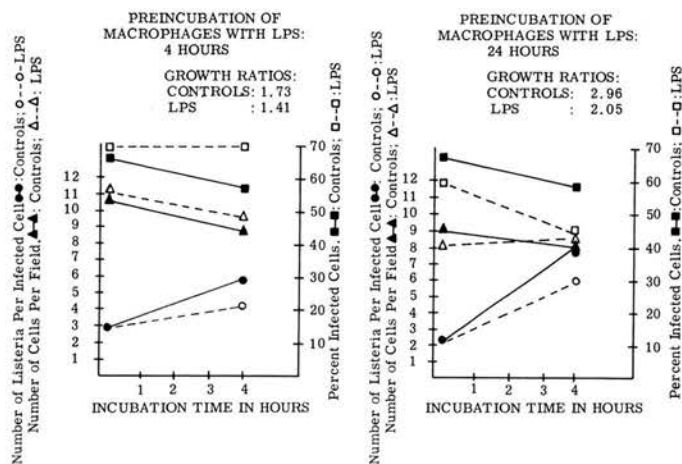


Figure 17. No antibiotics added. Same experiment as depicted in Figure 15.

percentage of infected cells tended to decrease during the incubation period. In control cultures this appeared to be mainly caused by the death of heavily infected cells as on these slides discrete clumps of bacteria could be seen surrounded by cellular debris apparently derived from a disintegrating cell. The decrease in percentage of infected cells on endotoxin-treated monolayers seems likely to be caused by disappearance of intracellular bacteria as no extracellular clumps of bacteria were seen or traces of disintegrating cells. The intracellular growth of L. monocytogenes was reduced in endotoxin-treated cells as compared with controls and there was some net killing of bacteria by macrophages exposed to endotoxin for 24 hours if antibiotics were added to the medium. All the differences between control cultures and endotoxin-treated cultures were much more marked after 24 hours of treatment as compared with 4 hours as is reflected in the growth ratios (shown in Figures 16 and 17). Experiments of this kind are clearly subject to considerable variation depending on the size of the bacterial inoculum in relation to the number of macrophages which it is difficult to adjust accurately.

Endotoxin had no direct effect on the viability of L. monocytogenes as was shown by adding endotoxin to cultures of L. monocytogenes and determining the viable count by the Miles and Misra method after 4 hours of incubation. Final concentrations of 10 and 100 $\mu\text{g/ml}$ of endotoxin gave a count of 2.3×10^9

bact./ml and 2.2×10^9 bact./ml respectively, when the control had 2.2×10^9 bact./ml (initial inoculum approximately 10^6 bact./ml).

These data indicate that endotoxin as used here caused activation of the macrophages in vitro as judged by increased ability to inhibit the growth of L. monocytogenes. This activation response was preceded by an increase in phosphatidyl-inositol turnover within the first 24 hours that was declining again before the bacteriostatic effect became marked.

5.2 Synthesis and secretion of lysosomal enzymes by macrophages exposed to various stimuli.

The changes in synthesis and secretion by macrophages of lysosomal enzymes in response to various stimuli were studied. The levels of three lysosomal enzymes, acid phosphatase, β -galactosidase and β -glucuronidase were measured in culture supernatants and cell lysates of macrophages following 6, 24 or 48 hours of culture thus estimating both release and intracellular concentrations of the enzymes. The results are summarized in Table 20. Both endotoxin and C. parvum with T-cells caused a significant increase in the release of all three enzymes after 24 and 48 hours of culture but not after 6 hours. There was no corresponding decline in intracellular levels indicating that synthesis was stimulated to the same degree as secretion. The synthesis of acid phosphatase by

Table 20:

The effect of various stimuli on the synthesis and secretion of lysosomal enzymes by macrophages.

Macrophages exposed to:	Lysosomal enzyme studied:	Optical density reading after incubation for:		
		6 hours	24 hours	48 hours
Endotoxin 60 µg/10 ⁶ cells	Acid phosphatase	S 0.002	0.023 ⁺	0.035 ⁺
		L 0.025	0.048 ⁺	0.052 ⁺
	β-galactosidase	S 0.003	0.013 ⁺	0.025 ⁺
		L 0.025	0.024	0.017
	β-glucuronidase	S 0.011	0.027 ⁺	0.037 ⁺
		L 0.034	0.044	0.050
<i>C. parvum</i> 100 bact./cell with T-cells 1/5 macrophages	Acid phosphatase	S 0.003	0.020 ⁺	0.028 ⁺
		L 0.025	0.045	0.031
	β-galactosidase	S 0.000	0.020 ⁺	0.031 ⁺
		L 0.022	0.029	0.017
	β-glucuronidase	S 0.001	0.021 ⁺	0.038 ⁺
		L 0.044	0.052	0.038
<i>C. parvum</i> 100 bact./cell	Acid phosphatase	S 0.001	0.013 ⁺⁺	0.019 ⁺⁺
	β-galactosidase	S 0.001	0.007	0.016 ⁺⁺
	β-glucuronidase	S 0.000	0.011 ⁺⁺	0.018
T-cells 1/5 macrophages	Acid phosphatase	S 0.001	0.002	0.009
	β-galactosidase	S 0.001	0.003	0.005
	β-glucuronidase	S 0.002	0.005	0.010
Latex 500 particles/cell	Acid phosphatase	S 0.001	0.002	0.002
		L 0.019	0.023	0.015
	β-galactosidase	S 0.006	0.007	0.007
		L 0.029	0.024	0.028
	β-glucuronidase	S 0.005	0.003	0.001
		L 0.022	0.027	0.036
Eagle's medium without serum	Acid phosphatase	S 0.001- 0.003	0.002- 0.007	0.001- 0.014
		L 0.023- 0.029	0.021- 0.054**	0.017- 0.038***
	β-galactosidase	S 0.000- 0.008	0.003- 0.009	0.005- 0.007
		L 0.017- 0.037	0.020- 0.025	0.017- 0.029****
	β-glucuronidase	S 0.001- 0.006	0.004- 0.011*	0.001- 0.010
		L 0.016- 0.048	0.027- 0.053	0.034- 0.051**

S: culture supernatant; L: cell lysate. *: Higher values in experiments testing for effect of endotoxin; **: higher values in experiments testing for effect of *C. parvum*; ***: lower values in experiments testing for effect of latex. ****: higher values in experiments testing for effect of latex. +: Significantly different from untreated control, $P < 0.001$; ++: $0.005 < P < 0.05$ (two-tailed Student's t-test). Standard errors of the means range from 0.001-0.005, highest for acid phosphatase in lysates of cells cultured 24 and 48 hours with endotoxin and supernatants of cells incubated 24-48 hours with *C. parvum* for all enzymes. The figures represent means of pooled data from 2-5 experiments, each performed in triplicates. All experiments using *C. parvum* and T-cells included controls with *C. parvum* or T-cells alone (only the supernatant levels measured). The effects of endotoxin and latex were tested in separate experiments.

endotoxin-treated cells was more markedly increased resulting in raised intracellular levels of this enzyme after 24 and 48 hours of culture. Macrophages exposed to latex or T-cells behaved like the untreated controls, showing very little release of lysosomal enzymes into the culture medium and fairly constant intracellular levels (the latter not measured in macrophages cultured with T-cells). Culture with C. parvum in the absence of T-cells gave inconsistent results and the raised values are mainly caused by the data from one experiment out of three and can probably be attributed to infection of the mice as noted before ("Results", 4.7). The stimulatory effect of C. parvum was, however, never as marked as that of endotoxin or C. parvum with T-cells, both of which yielded values of the same order.

These results form a pattern similar to that observed for the effect of the various stimuli on the turnover of phosphatidyl-inositol, e.g. endotoxin as well as C. parvum with T-cells are stimulatory whereas latex, T-cells or C. parvum without T-cells have no or minimal stimulatory activity. It will also be noted that no change had occurred in synthesis or secretion of lysosomal enzymes after 6 hours of culture the changes first becoming evident after the macrophages had been exposed to endotoxin or C. parvum with T-cells for 24 hours. In contrast

the uptake of myo-(2-³H)-inositol into endotoxin-treated macrophages was most markedly stimulated after 6 hours of incubation and declining again after 24 hours (see Figure 15). The time course of stimulated secretion of lysosomal enzymes is thus similar to that described above for the development of enhanced antibacterial activity.

DISCUSSION

DISCUSSION

1. MECHANISMS OF NON-SPECIFIC RECOGNITION BY MONONUCLEAR PHAGOCYTES.

1.1 The role of cell wall carbohydrates in the recognition of non-opsonized bacteria by macrophages.

1.1.1. Correlation between composition of bacterial surface carbohydrate and inhibition of bacterial binding to macrophages by sugars. The results presented here suggest that the binding of non-opsonized bacteria to mouse mononuclear phagocytes involves the recognition of carbohydrates in the bacterial cell wall or capsule by the phagocytes. This is illustrated by the close correlation found between the presence of a sugar in the cell wall or capsule of a bacterium tested and the ability of that sugar to inhibit the binding of the bacterium to the macrophage surface. The pattern of inhibition of binding of a micro-organism by sugars in relation to the composition of its cell wall or capsular carbohydrates was dealt with in detail in the "Results". It was noted that in most cases the binding of a bacterium was inhibited by all the monosaccharides that were constituents of its surface carbohydrates but was not affected by any other monosaccharides.

There were, however, a few exception to this pattern. Thus glucose failed to inhibit the binding of B. anthracoides although this sugar would be expected to be present in the cell wall teichoic acid. The binding of C. parvum was inhibited

by fructose and rhamnose, neither of which has been identified as a cell wall component, whilst two known components, mannose and N-acetylneuraminic acid had no inhibitory effect. Mannose did not inhibit the binding of the capsulated strain of K. aerogenes (NCTC 5055) despite being present in the capsule which appeared to mediate the binding of this organism. Mannose also failed to inhibit the binding of the smooth strain of S. typhimurium (SL 1542) which contains this sugar in the O-repeat unit of its lipopolysaccharide. The binding of the rough mutant SL 1096 was inhibited by rhamnose, a component of the O-repeat unit which this strain lacks. As mentioned in the "Results" this could be explained by the possibility that the mutant is "leaky". It is noteworthy that on three occasions mannose was found to be non-inhibitory even when present in the bacterial cell wall or capsule. This sugar is linked at branch points to other sugars both in the capsule of K. aerogenes (NCTC 5055) and the lipopolysaccharide of S. typhimurium (SL 1542) and may thus not be freely expressed and available for binding reactions. Alternatively the macrophage surface may lack the ability to bind this sugar. The inhibition of binding by sugars that are not present in the bacterial cell wall might be explained by a molecular similarity to a constituent sugar. Thus fructose is the keto-derivative of glucose; rhamnose, however, is the deoxy-derivative of mannose. The cell wall carbohydrate of C. parvum

was reported to contain "sialic acids" without further specification of the particular N-acyl derivative of neuraminic acid (Dawes et al., 1974).

1.1.2. Physical effects of sugars. The strict correlation found in most cases between the presence of a sugar in the bacterial surface carbohydrate and the ability of that sugar to inhibit binding of bacteria to the macrophage surface implies a specific chemical effect. Non-specific effects would not be expected to produce the pattern observed. Van Oss (1978) has attributed the inhibitory effect of glucose on the phagocytic activity of polymorphonuclear leucocytes to the influence of glucose on the cell shape. He observed that glucose at concentrations higher than 0.2% (11 mM) caused the withdrawal of pseudopods from the polymorphonuclear leucocytes and rendered them more spherical. This change in shape was thought to augment the negative surface charge of the cells and the electrostatic repulsion inhibiting the attachment of particles. The concentration of glucose used in the present experiments was just below this level (10 mM), no change in cell shape was observed under the conditions used and glucose only inhibited the binding of bacteria containing glucose in their cell wall or capsule, binding of glucose-deficient strains was not inhibited. Osmotic effects are not likely to have

occurred; care was taken to maintain iso-osmolality and the final concentration of sugars was low, only about twice the normal physiological concentration of glucose.

1.1.3. The role of carbohydrates in the adherence of bacteria to host cells. The importance of carbohydrates in biological recognition phenomena is being increasingly emphasised (Hughes & Sharon, 1978) and some examples of the role of surface carbohydrates in cellular adhesion were mentioned in the "Introduction" (8.1.8.). Of particular relevance here is the suggestion that surface carbohydrates may in some cases mediate bacterial adhesion to tissue cells and other surfaces (Anon., 1977; Costerton *et al.*, 1978). It was shown by Duguid & Gillies (1957) that mannose could inhibit agglutination of erythrocytes by E. coli and fimbriated strains of Shigella flexneri as well as the adhesion of the latter to colon epithelial cells. Similarly Old (1972) found that D-mannose, but not several other sugars tried, inhibited haemagglutination by fimbriated Sh. flexneri and S. typhimurium and that the hydroxyl groups on carbon atoms 2,3,4 and 6 of the mannose were of particular importance for the effect. The biochemistry of adherence of E. coli has recently been studied in some detail. It has been shown that the presence of D-mannose in low concentrations (down to micromolar levels) in the medium can inhibit the binding of piliated E. coli to

human oral epithelial cells (Ofek et al., 1977) green monkey kidney cells (Vero) (Salit & Gotschlich, 1977) mouse peritoneal macrophages and human polymorphonuclear leucocytes (Bar-Shavit et al., 1977). In contrast D-mannose had no effect on the binding of piliated E. coli to human urinary tract epithelial cells (Svanborg Edén & Hansson, 1978) suggesting that several factors may contribute to the tissue adherence of E. coli. Haemagglutination by isolated K88 antigen from E. coli strains causing piglet diarrhoea can be inhibited by β -D-galactosyl-containing glycoproteins but not by simple sugars (Gibbons et al., 1975).

There is now some evidence to indicate that E. coli can recognize mannose-residues on the surface of host cells. Thus E. coli is agglutinated by the mannose polymer mannan and a substance that binds mannose and inhibits binding of E. coli to oral epithelial cells can be extracted from the bacteria (Ofek et al., 1977). Preincubation of E. coli with mannan inhibited attachment to phagocytic cells but preincubation of the phagocytes with this substance had no effect (Bar-Shavit et al., 1977). The same workers also found that the binding of S. typhi to mouse peritoneal macrophages was inhibited by the presence of D-mannose but not L-fucose. These results clearly contrast somewhat with those from the present study. The concentrations of sugars used were similar or higher (2 mg/ml = 11 mM and 20 mg/ml) but the main difference is that the sugar

was added with the bacteria (incubated for 30 minutes at 4°C). The failure of mannan to inhibit attachment of E. coli when preincubated with the phagocytes is, however, in agreement with the present findings that preincubation of mannose failed to inhibit the binding of three mannose-containing bacteria. The differences can thus be explained on the basis of different viewpoints of observation, in one case the recognition of host cells by bacteria was tested whilst the present study dealt with the recognition of bacteria by host phagocytic cells. It seems likely that mutual recognition may be taking place between bacteria and phagocytes in a similar way as must be the case in cell adhesion phenomena involving cells of the same kind (see "Introduction" 8.1.5., 8.1.7., 8.1.8.).

Studies with Vibrio cholerae have yielded results akin to those just described for E. coli. Thus haemagglutination and adhesion to intestinal brush border membranes by V. cholerae was inhibited by the presence of very low concentrations (1-10 µg/ml) of L-fucose. V. cholerae also attached to L-fucose-coated agar beads (Jones & Freter, 1976). The inhibitory effect of L-fucose reached a plateau of approximately 60% at 100 µg/ml indicating that fucose-mediated binding was not the only adhesive mechanism operating. The same authors have also noted that L-fucose did not inhibit the binding of V. cholerae to slices of intestinal mucosa where the mucus gel was present,

again indicating that bacterial adherence is probably often a complex process (Freter & Jones, 1976).

Bacterial polysaccharides have been implicated as mediators of bacterial adhesion. Thus Gibbons and van Houte (1975) have demonstrated that glucans produced by Str. mutans are important in the attachment of this organism to teeth. Similarly such glucans promote the binding of Streptococci to damaged heart valves in vitro (Ramirez-Ronda, 1978). The production of an extracellular polysaccharide appears to enhance the binding of Rhizobium leguminosarum to the roots of pea plants (Sanders et al., 1978). The specificity of attachment of different Rhizobium species to their host plants has in previous reports been attributed to surface structures rather than the secreted exopolysaccharide. Thus lectins extracted from seeds of leguminous plants have been reported to bind to polysaccharides isolated from Rhizobial lipopolysaccharide (Planqué & Kijne, 1977) or capsules (Dazzo & Hubbell, 1975) and in a highly specific way to O-antigen-containing lipopolysaccharide of symbiont Rhizobial strains (Wolpert & Albersheim, 1976). Dazzo and Hubbell (1975) demonstrated antigenic cross-reactivity between the capsular Rhizobium antigens and the surface of epidermal cells of the clover. They proposed a model whereby the clover lectin, trifoliin, forms a specific link between the root surface and the bacterial capsule (Dazzo & Hubbell, 1975; Dazzo et al., 1978). The adherence of Lactobacilli to chicken crop epithelial

cells appears to depend on bacterial surface carbohydrate as it is markedly decreased following pretreatment of the bacteria with periodate or Concanavalin A (Fuller, 1975). Costerton et al. (1978) have developed the hypothesis that many different bacteria attach to living and dead surfaces by means of a loosely attached carbohydrate coat, the "glycocalyx". In the case of adherence to living cells this is seen to involve binding of the "glycocalyx" to the exposed carbohydrates in the cell (its "glycocalyx") thus coming back to the idea of mutual recognition of carbohydrate-containing structures.

The lipoteichoic acid of the cell wall of group A Streptococci may also be involved in the binding to host cells. This substance if isolated from the bacteria binds to erythrocytes and buccal epithelial cells (Ofek et al., 1975; Allan & Beachey, 1978) and can block the adherence of group A Streptococci to epithelial cells (Ofek et al., 1975). Streptococci denuded of their lipoteichoic acid by exposure to penicillin can no longer bind to erythrocytes or epithelial cells (Allan & Beachey, 1978). The lipid moiety of lipoteichoic acid appeared to be particularly important in these binding reactions but the role of sugars was not specifically investigated. Lipoteichoic acids of Gram-positive bacteria are composed of teichoic acid attached to a glycolipid and are usually deeply embedded in the cell wall but some Streptococcal species appear to produce extracellular lipoteichoic acids (see Ghuysen, 1977).

1.1.4. Model of carbohydrate-mediated binding of bacteria to macrophages. In the present experiments it is noteworthy that most of the inhibitory sugars caused a decrease in bacterial binding to macrophages of about 50%. The simplest explanation for this finding would perhaps be that even if the binding sites for one sugar are blocked binding can still take place via the other sugar components of the cell wall. A variation of this explanation is that the strength of binding is directly correlated with the number of sugars involved so that blocking of the binding site for one sugar would result in reduced strength of binding and the bacteria would be more easily removed by washing which would be reflected in a decreased proportion of cells with bound bacteria. This type of explanation gains some support from the results obtained with the rough strain of S. typhimurium that possessed only the inner core of the lipopolysaccharide (SL 1102). The binding of this strain was more strongly inhibited by lipid A than was usually observed with the sugars. This and KDO (keto-deoxyoctonate) were the only components of the lipopolysaccharide remaining. This would be the expected result in light of the interpretation given above as there would be no other surface structure apart from KDO available for binding when binding sites for lipid A have been blocked. Binding of the other strains of S. typhimurium was less strongly inhibited by lipid A. In these strains other sugars can mediate the binding and as the

carbohydrate-chain attached to the lipid A gets longer lipid A will be less accessible for attachment.

Another possible explanation for the degree of binding still occurring after the exposure of the macrophages to an inhibitory sugar is displacement of bound monosaccharides by bacteria. Binding experiments performed in the presence of the inhibitory sugars might have given stronger inhibition if this was the case but such a procedure would not have distinguished between inhibition of binding sites on the macrophages and possible binding sites on the bacterial surface. This type of explanation does not account readily for the results obtained with inhibition of binding of different strains of S. typhimurium by lipid A as outlined above. It could rather have been expected that the rough strain expressing lipid A most freely was more efficient at competing with macrophage-bound lipid A than the other strains and binding of this strain, therefore, least affected by preincubation with lipid A. As already stated the opposite result was obtained.

A third alternative is that other recognition mechanisms were involved along with recognition of surface carbohydrates. This would imply that blocking with only one sugar would more or less inhibit any carbohydrate-mediated binding and that the residual binding observed was mediated by other mechanisms. If this was the case blocking with sugars would hardly have been expected to produce the well-defined inhibition patterns observed

according to the carbohydrate-composition of the bacteria. Inhibition experiments using two or more sugars simultaneously might clarify this point.

Figure 18 (p. 299A-D) shows in a very schematic and simplistic way how the carbohydrate-mediated binding of bacteria to macrophages and inhibition of binding by monosaccharides might be visualized. The sugars are shown arranged in an ordered fashion on the bacteria whilst the arrangement of binding sites for monosaccharides on macrophages is random (derived for the figure from tables for random sampling numbers). The sketch bears, of course, no relation to actual sizes and is not meant to represent any particular number of bacteria or macrophages but might rather be seen as a reflection of frequencies of sugars and binding sites. If it is postulated that binding of two monosaccharides at least is needed for bacterial adherence to occur the 100 monosaccharide binding sites on the macrophages shown offer 23 separate possibilities for the attachment of bacterium A (Figure 18 a). Blocking of binding sites for monosaccharide 1 (green, Figure 18 b) reduces the possibilities to 9, blocking with monosaccharide 2 (red, Figure 18 c) leaves 11 and blocking with monosaccharide 3 (blue, Figure 18 d) 8 separate possibilities. The inhibition of bacterial binding is thus 50-60% for each sugar. In the Figure it was assumed (partly for the sake of clarity) that blocking of the binding site for one sugar would inhibit binding to directly adjacent

binding sites for the other two sugars. If this was not the case the two bacterial attachment sites in the upper left hand corner (for example) would not be completely blocked if only one of the monosaccharide binding sites was blocked and the number of possibilities for bacterial binding with binding sites for sugar 1, 2 or 3 blocked would increase to 12, 17 and 13 respectively (25-50% inhibition of binding).

If binding of bacteria is significantly promoted by simultaneous binding of more than one sugar (as assumed in the figure) this would tend to even out differences in inhibition patterns that might be caused by unequal frequencies of binding sites for different sugars. A requirement for attachment of more than one sugar would also lead to more effective inhibition of binding by only one sugar by an indirect effect as compared with the assumption that blocking of the binding site for one sugar had no influence on binding of the other sugars on the bacterial surface.

Figures 18 e and f shows the binding and inhibition of binding of an organism (bacterium B) that does not contain monosaccharide 3 (blue). The binding of this organism is shown not to be affected by blocking of binding sites for monosaccharide 3; here it must be assumed that the other sugars can approach the blocked binding site. There are 19 separate possibilities for the attachment of bacterium B and these are reduced to 6 (or 7 if the block does not interfere with binding

to adjacent sites) by blocking of binding sites for monosaccharide 1 (green).

The figure may thus help to illustrate how the blocking of a binding site for one monosaccharide could inhibit carbohydrate-mediated binding of bacteria to macrophages to the degree observed in the present experiments.

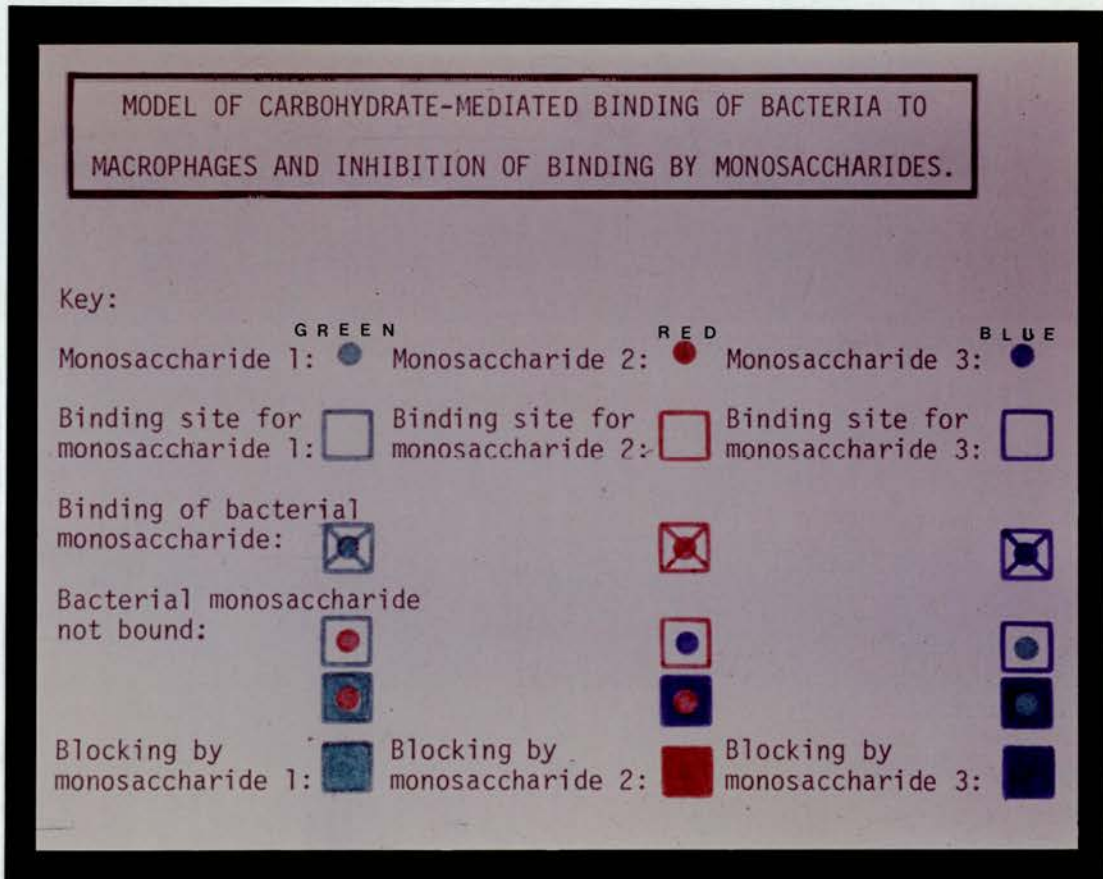
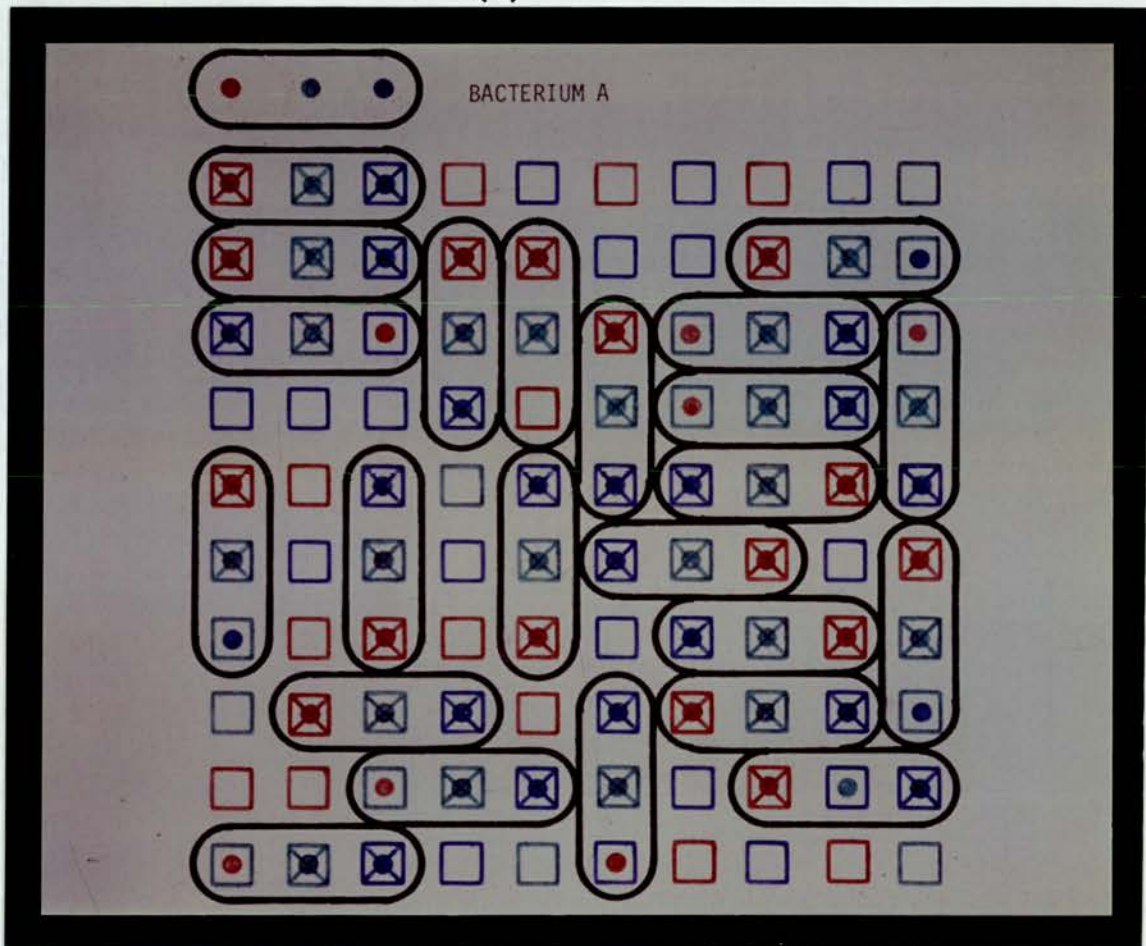
Key to Figure 18.

Figure 18 (contd.)

(a)



(b)

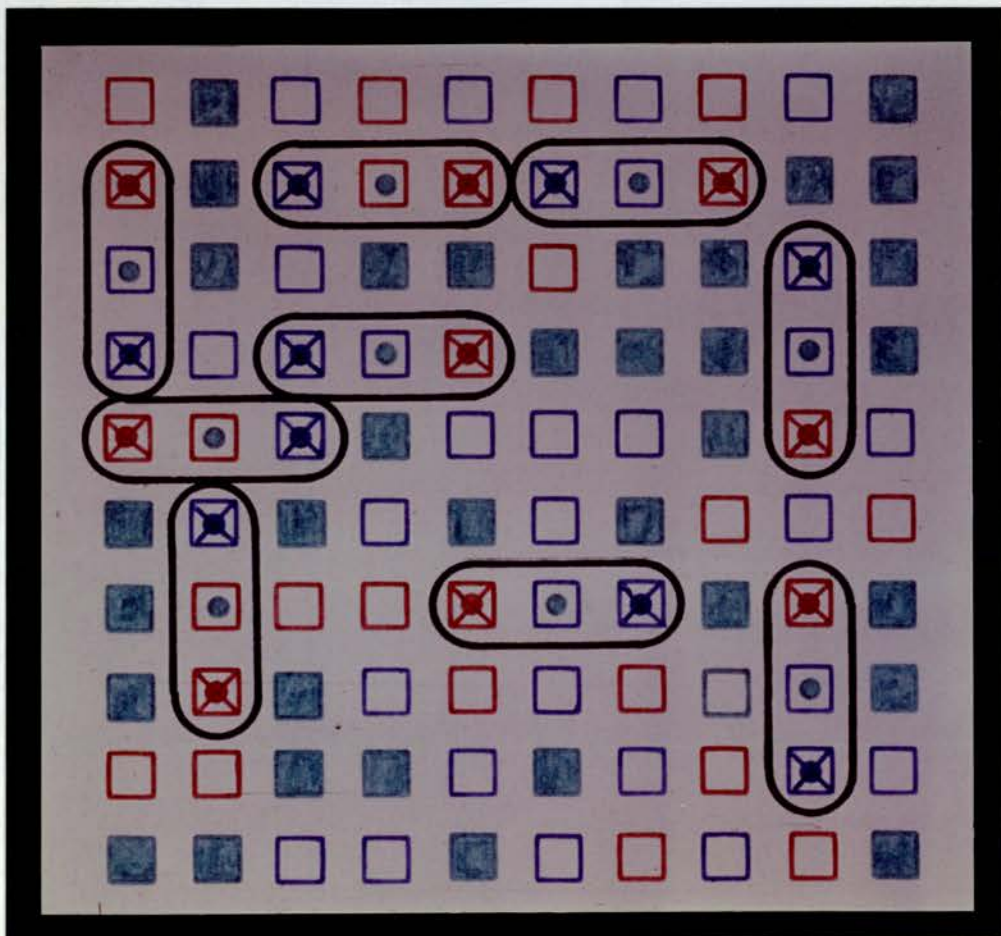
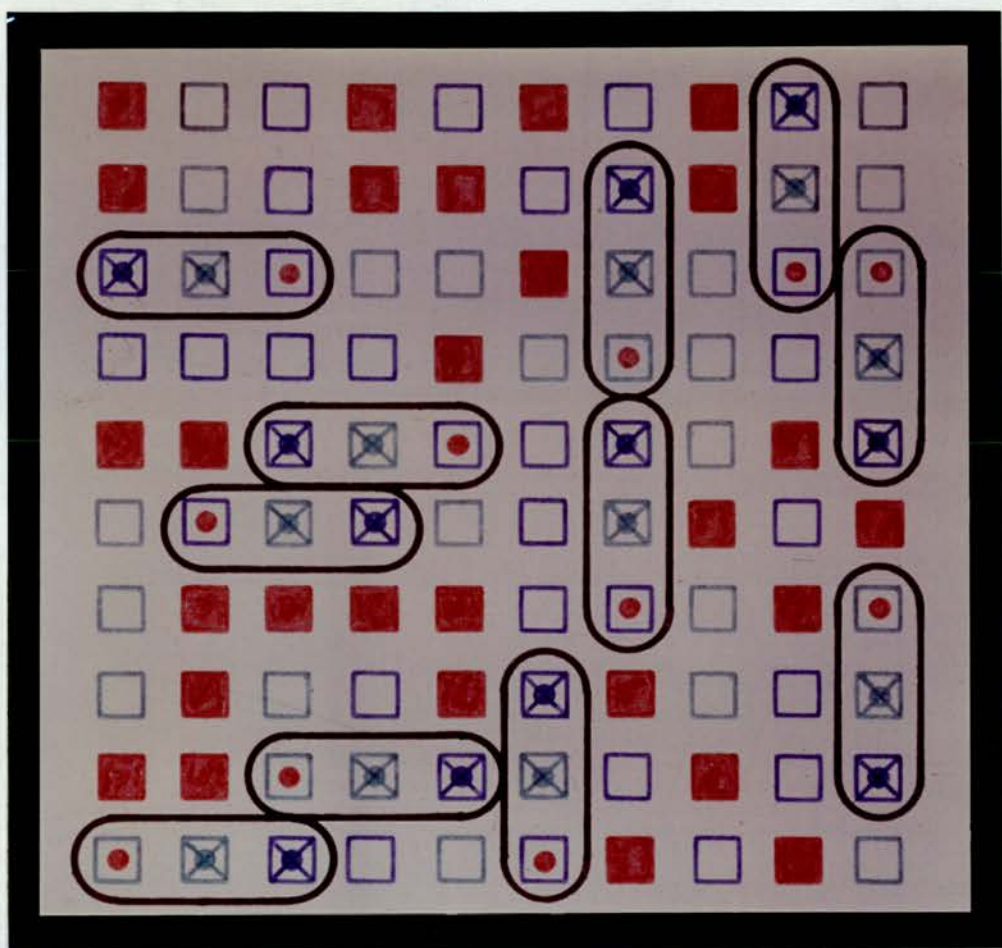


Figure 18 (contd.)

(c)



(a)

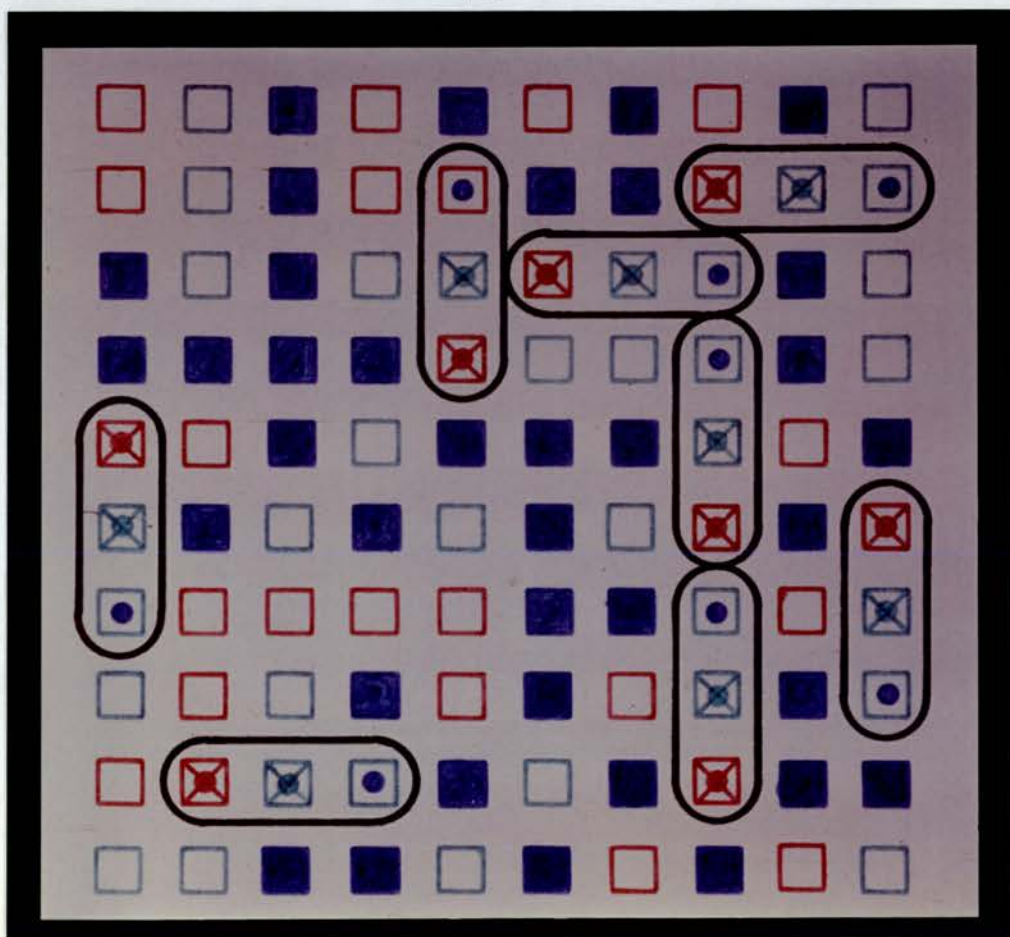
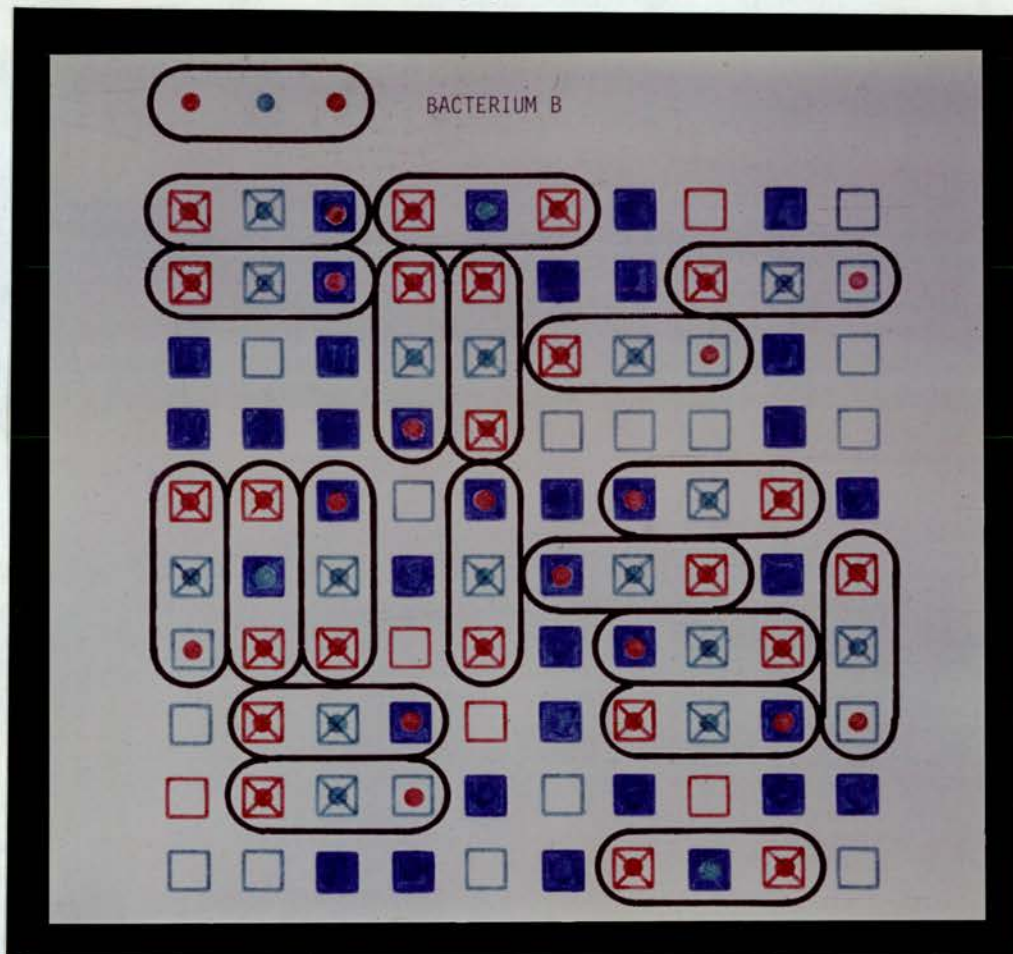
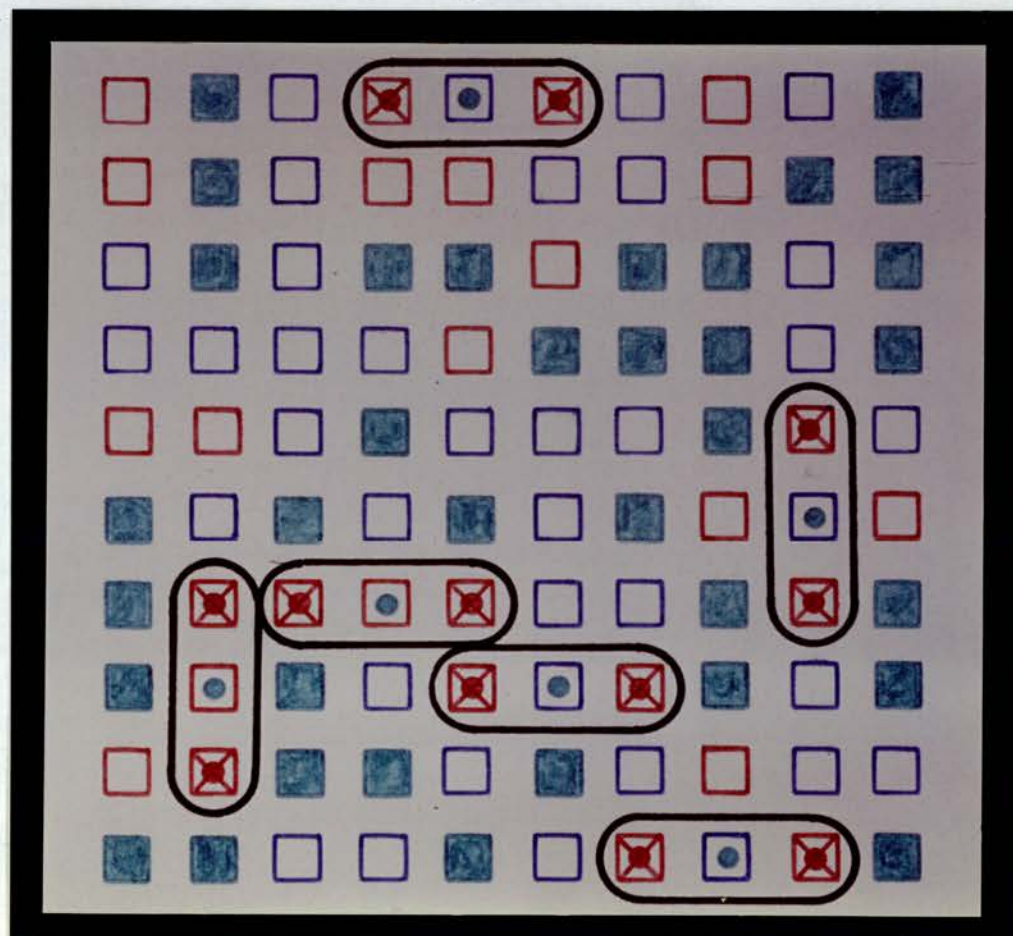


Figure 18 (contd.)

(e)



(f)



1.2 The nature of plasma membrane components of macrophages involved in the binding of non-opsonized bacteria.

1.2.1. Binding not mediated by cytophilic antibody.

The binding of non-opsonized C. parvum to macrophages was shown not to be mediated by cytophilic antibodies on the macrophage surface as mild treatment with trypsin followed by brief recovery in serum-free medium had no effect on the binding of C. parvum but did remove added cell-bound antibodies.

1.2.2. The reported effects of enzyme treatment on surface functions of macrophages. The macrophages were subjected to treatment with enzymes and periodate in an attempt to gain some information on the nature of plasma membrane components involved in the recognition of non-opsonized bacteria. It was found that the binding of C. parvum was decreased to about the same degree (around 50%) directly following pre-treatment of the macrophages with two proteolytic enzymes (trypsin and pronase), three phospholipases (A, C and D) and one glycosidic enzyme (β -galactosidase). Exposure to periodate had a similar effect. When the macrophages had been pre-treated with neuraminidase an increase in binding was observed.

For comparison, some of the reported effects of enzyme treatment on some macrophage functions presumably involving the plasma membrane are summarized in Table 21 (p. 317) which also includes the relevant references. Although the range of enzymes used

in testing for effects on a specific function was often limited it appears that the more specific receptors that are often regarded as defined molecular entities are destroyed selectively by a few enzymes but not affected by others. Thus the Fc-receptor has been found to be destroyed by phospholipases but binding of immunoglobulins was not affected or slightly increased following treatment with trypsin and somewhat increased after exposure to neuraminidase (for references see the table). The separate Fc-receptor on mouse macrophages that binds only IgG2a is, however, destroyed by trypsin. A polypeptide that binds aggregated IgG has recently been isolated from cells of the macrophage-like cell line P388D₁. This polypeptide has a low carbohydrate content and is not sensitive to the action of trypsin (D'Urso-Coward & Cone, 1978). The receptor for complement is destroyed by trypsin but the effect of other enzymes on this receptor has not been studied. Binding of MIF is decreased following exposure to α -L-fucosidase as well as trypsin and chymotrypsin and as mentioned in the "Introduction" (8.2.7.) fucose appears to be an essential component of the receptor for MIF. Treatment with neuraminidase increases binding of MIF as was seen with IgG. Induction of macrophage cytotoxicity by a lymphokine (MAF) was not affected by pre-treatment of the macrophages with trypsin or papain but was inhibited when the macrophages had been exposed to pronase.

The incubation period with the lymphokine in these experiments was 18 hours and it is therefore possible that some recovery of membrane receptors occurred during that period. It is noteworthy in this respect that according to the present study pronase could not be removed from the cells by washing and could thus have prolonged effects. The results cited above seem to indicate that receptors for lymphokines are of glycoprotein nature. It has, however, recently been suggested on the basis of experiments using isolated glycolipids from macrophages and other cells that the receptor for MIF might be a glycelipid (Higgins et al., 1978).

The findings on IgG receptors and the receptor for MIF indicate that results obtained by using enzyme treatment of intact cells must be interpreted with caution. They may not give direct information on the nature of receptors and perhaps may sometimes indicate secondary requirements for receptor function.

Treatment of human monocytes with phospholipases decreased their chemotactic response towards casein whilst exposure to trypsin, neuraminidase or α -L-fucosidase had no effect. It was speculated that the chemotactic activity of casein was based on its hydrophobicity and that it reacted with the lipid bilayer (Wilkinson, 1976a). The table also contains the limited information available on the effect of enzyme treatment

on binding or phagocytosis of three unopsonized particles. Thus the binding of Mycoplasma pulmonis was not affected by either trypsin or neuraminidase but binding of chemically modified erythrocytes as well as heterologous erythrocytes and zymosan was diminished following exposure to trypsin which is in agreement with the present study. Treatment with neuraminidase led to increased phagocytosis of negatively charged plastic microspheres by human monocytes.

1.2.3. The effect of enzyme treatment on the binding of non-opsonized bacteria to macrophages. The similar effects of several different enzymes obtained in the present study contrast somewhat with the results listed in Table 21. It appears that many different surface components contribute to a similar degree to the binding of non-opsonized bacteria either synergistically or separately.

From studies on the effects of enzyme treatment on other plasma membranes it is known that proteolytic enzymes only cleave off protein- or glycoprotein-chains that extend beyond the outer lipid layer in the intact cell (Steck et al., 1971). It is possible that such treatment could expose components that might still contribute to a limited extent in a non-specific recognition phenomenon although they might not be able to substitute for more specific receptors.

Modification of the phospholipid components of the plasma

membrane may have indirect effects. Thus many membrane enzymes are dependent on the presence of certain phospholipids (Singer & Nicolson, 1972; Coleman & Bramley, 1975; Low & Finean, 1977 & 1978). It is of interest to note here that the activity of some glycosyltransferases has been shown to be abolished following treatment with phospholipases and could be partially restored again by the addition of phospholipids (Labow et al., 1973). This class of enzymes has been implicated in cellular adhesion phenomena involving surface carbohydrates (see "Introduction", 8.1.8.). Treatment of intact cells with phospholipases only affects the outer leaflet of the lipid bilayer (Zwaal et al., 1975) and this results in distortion of the plasma membrane (Coleman et al., 1970; Allan et al., 1975). It seems possible that such distortion changes the cellular surface architecture in a way that interferes with recognition and adherence.

With reference to Figure 18 in the preceding chapter it is conceivable that the binding sites for different monosaccharides could vary in their susceptibility to the different enzymes so that even if the binding site for one monosaccharide was destroyed by an enzyme other monosaccharide residues would still be bound.

Neuraminidase was the only enzyme that caused an increase in the binding of C. parvum to macrophages. This is likely to have been caused by a decrease in the negative surface charge

as it is known that sialic acids are the major contributors to the negative surface charge of eukaryotic cells (Eylar et al., 1962; Weiss et al., 1972) and the cell wall of C. parvum appears to be negatively charged (Dawes et al., 1974). It can be seen from Table 21 that treatment with neuraminidase has previously been found either to increase both specific and non-specific recognition activity by macrophages or leave it unaffected; a decrease in activity has not been reported. As mentioned above treatment with neuraminidase has been shown to lead to an increase in phagocytosis of negatively charged plastic microspheres by human monocytes (Weiss et al., 1966) whilst phagocytosis by polymorphonuclear leucocytes of uncharged starch particles was not affected (Noseworthy et al., 1972). Exposure of green monkey kidney cells to neuraminidase has also recently been found to enhance the adherence of E. coli to these cells (Salit & Gotschlich, 1977).

It is unlikely that the enzymes exerted their effects in the present experiments by binding to the cell surface and thereby blocking the attachment of bacteria. The enzymes were washed away before adding the bacterial suspension and the successful removal of the enzymes, apart from pronase, is indicated by the recovery of normal binding capacity during one hour in tissue culture medium after the washing. This recovery is unlikely to have been caused by internalization of

surface-bound enzymes as the different effects of EDTA on recovery would then have to be explained by interference with the internalization of some of the enzymes but not others.

One explanation that must be considered for the similar effects of several different enzymes is that these were to some extent caused by contaminating enzymes as it is known that commercially available enzymes do contain impurities (Zwaal et al., 1975). These would not have represented more than a few per cent of the concentration of an enzyme used and as the changes observed were only about 50% of normal values low concentrations of contaminating enzymes would not be expected to have had a marked effect. Although the effects of different enzymes appeared to be similar when treated macrophage monolayers were tested directly after exposure to the enzymes differences in their effects were revealed by the results of the recovery experiments (see below). Morphological differences in cells exposed to different enzymes were also observed. Thus cells treated with phospholipases showed smoother surface outlines than normal cells or cells treated with the other enzymes. There are, therefore, some indications that specific enzyme effects were occurring.

1.2.4. The effect of treatment with periodate on the binding of non-opsonized bacteria to macrophages. Mild treatment with periodate produced a similar effect on the binding

of C. parvum to that caused by exposure to enzymes. This could to a large extent be reversed by subsequent brief exposure to borohydride which results in reduction of the aldehyde group produced by the action of periodate on carbon atom 7 on neuraminic acid back to an alcohol group. It appears, therefore, that the aldehyde group interferes with binding and/or that the hydroxyl groups on carbohydrates take part in the binding reaction. Unmodified hydroxyl groups appear to be important in carbohydrate-mediated recognition phenomena such as the binding of lectins (Sharon & Lis, 1972) and haemagglutination by fimbriate enterobacteria (Old, 1972). The results obtained using periodate might seem to indicate that intact sialic acid residues are relatively important in the binding of C. parvum especially since the effect of periodate was not completely reversed by the action of borohydride. However, treatment with neuraminidase in order to remove sialic acids caused an increase in binding as mentioned above. Sialic acids are usually linked to galactose in glycolipids and glycoproteins (Tuppy & Gottschalk, 1972) and this sugar would be exposed following treatment with neuraminidase. The depressing effect of β -galactosidase on the binding of C. parvum indicates that galactose residues on the macrophage surface are involved in the attachment of the bacteria. The finding of Wollweber & Fritsch (1975) that Ricinus communis

agglutinin, which is specific for galactose, binds to untreated mouse peritoneal macrophages indicates that this sugar is readily accessible on the macrophage surface. As regards the effects of changing or removing sialic acid residues this is likely to alter the balance between repulsive and attractive forces in cellular adherence reactions. When sialic acids are removed the result is a diminution in repulsion caused by their negative charge. After treatment with periodate the negative charge of the carboxyl group on carbon atom 1 will be retained but there is a decrease in the number of hydroxyl groups which may contribute to adhesion.

Exposure of oral epithelial cells to periodate was shown by Ofek et al. (1977) to inhibit subsequent binding of E. coli. Although the concentration used was ten times that used in the present study it is not likely to have been high enough to affect surface sugars other than sialic acids. The mannose residues on the epithelial cells, which on other evidence were likely to mediate the attachment of E. coli (see above), would probably have been unchanged and the effect of periodate treatment of a more indirect nature. Treatment of macrophages with low concentrations of periodate (1-10mM) enhances their response to MIF (Remold, 1977) which seems to bind to fucose on the macrophage surface (Remold, 1973). This was interpreted as an inhibitory effect on a surface esterase that would normally regulate the response to MIF (Remold, 1977).

1.2.5. Recovery from the effects of enzyme treatment.

In the present study the macrophages were shown to recover normal binding capacity if they were left for one hour in tissue culture medium without serum after the enzymes had been washed away.

Cells treated with pronase were an exception; this enzyme could apparently not be removed by washing as its effects were more marked after incubation for one hour in culture medium than directly following the incubation with the enzyme solution. The observed rate of recovery was more rapid than that reported by some other workers for recovery of various surface functions of macrophages following phagocytosis or treatment with trypsin. Thus Lagunoff (1971) found that macrophages needed 4 to 6 hours to resume normal pinocytic activity following treatment with trypsin (1mg/ml) for 30 minutes. Werb (1975) has reported a recovery period of 5 to 8 hours following treatment with trypsin when testing for phagocytosis of aldehyde-treated red blood cells. The concentration of trypsin used, or incubation period with the enzyme was not stated but the same author has previously used concentrations ranging from 0.5 to 10 mg/ml for 60 minutes (Werb & Cohn, 1971b). Czop et al. (1978) have recently observed an even longer recovery period of 48 hours when testing the phagocytosis of rabbit and mouse erythrocytes by human monocytes. The concentration of trypsin required was very low, 5 μ g/ml,

but the incubation with the enzyme was longer (30 minutes) than that used in the present study. By contrast Ilgen & Burkholder (1974) have described that a C4-determinant reappears on the macrophage surface within minutes following removal by exposure to trypsin, 0.5 mg/ml for 15 minutes. (See also "Introduction", 7.2.2. & 7.2.3.).

The concentration of trypsin used and length of treatment may be of importance in determining the extent of the effect and milder conditions may result in defects that are more rapidly repaired than those caused by more prolonged action or higher doses. Different surface structures may also vary in their relative susceptibility to enzyme attack. Thus fat cells lose their ability to respond to insulin or glucagon following exposure for one minute at 37°C to 1 mg/ml of trypsin. Responsiveness to insulin was restored to almost normal one hour after stopping the enzyme action; this recovery required protein synthesis. Recovery of responsiveness to glucagon took longer (Kono, 1969b). An even lower concentration of trypsin (10 µg/ml) applied to fat cells for 15 minutes resulted in loss of affinity for insulin without a change in the number of insulin receptors or metabolic responsiveness to insulin. There was no return to normal affinity after 4 hours (Cuatrecasas, 1971).

Phagocytosis of large numbers of particles in vitro has

been found to result in depressed activity of the Fc-receptor of monocytes (Schmidt & Douglas, 1972) and binding of Concanavalin A to macrophages (Lutton, 1973) for 4 to 6 hours. Return to normal was in both cases found to require protein synthesis.

There is some evidence for quite rapid synthesis and regeneration of macrophage plasma membrane components. Skutelsky & Hardy (1976) reported full regeneration of an anionic surface component within three hours following removal by cationic ferritin. The incorporation of labelled amino acids into macrophage plasma membrane proteins reaches a peak after 90-150 minutes (Nachman et al., 1971b).

Recovery from the effects of enzyme treatment was influenced by the availability of divalent cations in the medium. Thus recovery following treatment with trypsin and β -galactosidase was prevented by the presence of 10 mM EDTA whilst return to normal following exposure to the phospholipases was only slightly affected and in the case of neuraminidase treatment recovery was unaffected by EDTA. Binding to pronase-treated cells was higher following a recovery incubation with EDTA than in normal medium and this was similar to the binding recorded directly following exposure to the pronase solution. It is known that some of the components of pronase require Ca^{++} for their stability (Awad et al., 1972) and the EDTA would thus

have prevented the continued effect of this enzyme which remained cell-bound after the washing procedure. The varying susceptibility of recovery to removal of divalent cations according to the enzyme used might imply the existence of separate repair mechanisms with different requirements for divalent cations. It is interesting to note here the demonstration by Wang et al. (1976b) of the enzyme cytidine diphosphocholine phosphotransferase in the macrophage plasma membrane which might enable macrophages to repair damaged plasma membrane phospholipids in situ (see also "Introduction" 7.1.7. & 7.2.2.). Canonico et al. (1978) have recently demonstrated the occurrence of galactosyl transferase and N-acetylglucosaminyl transferase in isolated macrophage membranes. Their experiments did not allow firm distinction between the Golgi apparatus and the plasma membrane as the main site of these glycosyltransferases but the plasma membrane was felt to be the more likely site. It can be speculated that the damage produced by β -galactosidase in the present experiments could be repaired by a surface galactosyl transferase. It is known that many glycosyltransferases require divalent cations for their action (Roseman, 1970). Interestingly sialyl transferases appear to function independently of divalent cations (Roseman, 1970).

Little is known about the mechanisms involved in the repair of plasma membrane damage. Some workers have concluded that enzymatic damage elicits no specific repair processes but that

the plasma membrane is restored to normal by its ordinary turnover (see Cook, 1977). This conclusion was based on work with continuous cell lines that have lost their differentiation and show a more uniform surface than normal, freshly isolated tissue cells. It is possible that in normal cells functionally different areas of the plasma membrane may be turned over at different rates and that damaged components stimulate selective rapid replacement (Tweto & Doyle, 1977). Studies with phagocytosing macrophages have shown that certain macrophage surface components can segregate into separate areas (see "Introduction", 7.2.3.). The results obtained here showing different sensitivity of recovery to removal of divalent cations depending on the enzyme used do not agree with repair by simple bulk replacement alone and tend to suggest that separate repair processes are operating.

1.2.6. Absence of recovery from the effects of periodate. It is noteworthy that there was no sign of return to normal binding activity when macrophages that had been exposed to periodate were incubated for one hour in tissue culture medium. This was not caused by a failure to remove periodate by washing as the presence of the chemical during one hour would have resulted in cell damage. One possible explanation is that the damage brought about by the mild periodate treatment was too subtle to stimulate the repair processes involved in the recovery from the effects caused by

enzymes. It has been shown that glycoproteins containing sialic acids that have been modified by exposure to periodate and borohydride have the same half life in vivo as unmodified glycoproteins (Van Lenten & Ashwell, 1971).

1.2.7. The effect of antibodies against membrane determinants on surface attachment and phagocytosis. It can be seen from the foregoing discussion that the information gained from studies using enzymatic or chemical treatment of intact cells about plasma membrane components involved in surface functions of cells may be incomplete and sometimes perhaps misleading. A related approach is the use of antibodies directed against cell surface components. Thus Schroit & Gallily (1977) have studied the effects of anti-H-2 antisera and anti-macrophage serum on binding of particles and phagocytosis by mouse peritoneal macrophages. The anti-H-2 antisera were found to depress both binding and phagocytosis of antibody-coated erythrocytes and binding of Shigellae whilst phagocytosis of Shigellae and latex particles was unaffected. Anti-macrophage serum caused decreased binding and phagocytosis of both the sensitized erythrocytes and Shigellae but phagocytosis of latex particles was again not changed. This was interpreted by the authors to imply the existence of at least three different phagocytic recognition sites. With reference to the "zippering" theory of phagocytosis (see "Introduction", 9.2.1.)

differences in size of the test particles and the number of determinants on the macrophage surface covered by the antisera might have contributed to the results obtained. The anti-macrophage serum would be expected to react against a greater number of determinants as compared with anti-H-2 and thus interfere more effectively with surface binding. Phagocytosis of larger particles such as red blood cells would be more easily disturbed by surface-attached molecules.

1.2.8. Isolation of membrane molecules. The most direct approach to identifying plasma membrane components involved in surface function is to isolate plasma membrane molecules and test their activity. It was mentioned in the "Introduction" (8.1.2.) that some hormone receptors have now been isolated from plasma membranes. A molecule with Fc-receptor activity has been isolated from cells of the macrophage-like cell line P388D₁ (see above). It was recently reported that fibronectin, a plasma membrane protein active in cellular adherence phenomena binds to St. aureus (Kuusela, 1978) and this is the first example of a mammalian plasma membrane glycoprotein binding to a bacterium. The binding was inhibited by glucosamine and galactosamine; divalent cations were not required for binding. Pearlstein et al. (1978) have fractionated macrophage plasma membrane proteins on SDS-polyacrylamide gel electrophoresis. They failed to detect any fibronectin and

found that these macrophage plasma membrane proteins were relatively resistant to the action of trypsin.

1.2.9. Concluding remarks. Taken together the results obtained in this study with enzymatic and chemical treatment of macrophages and the circumstantial evidence discussed tend to favour a more important direct role for plasma membrane glycoproteins in the binding of non-opsonized bacteria to macrophages than the plasma membrane phospholipids which probably play an auxilliary role. The evidence is obviously not conclusive. If the surface glycoproteins are mainly involved this would mean a recognition reaction between the surface carbohydrates on the macrophages and bacterial cell wall carbohydrates.

Table 21. Reported effects of enzyme treatment on macrophage function.

Enzyme	Function tested			Reference
	Decreased	Unchanged	Increased	

Lipolytic enzymes:

Lipase

Phospholipase A

Binding of cyto-
philic IgG

(1)

Phospholipase C

Binding of anti-
gen-antibody
complexes

(2)

Chemotaxis towards
casein

Phospholipase D

Binding of cyto-
philic IgG

(1)

Sphingomyelinase

Chemotaxis towards
casein

(3)

Proteolytic enzymes:

Trypsin

Binding of Myco-
plasma pulmonis

(4)

Table 21. Contd.

Enzyme	Function tested			Reference
	Decreased	Unchanged	Increased	
Trypsin (contd.)			Binding of anti-gen-antibody complexes	(2)
			Binding of cytophilic IgG	(1)
		Induction of cytotoxicity by MAF; cytotoxic effect		(5)
	Binding of complement-coated particles	Binding of antibody-coated particles		(6)
	Binding of complement-coated particles			(7)
	Binding of and response to MIF			(8)
	Binding of IgG2a			(9)
	Binding of chemically altered erythrocytes			(10)

Table 21. Contd.

Enzyme	Function tested			Reference
	Decreased	Unchanged	Increased	
Trypsin (contd.)	Phagocytosis of zymosan and heterologous erythrocytes			(11)
		Chemotaxis towards casein		(12)
Chymotrypsin			Binding of cytophilic IgG	(1)
	Binding of and response to MIF			(8)
Papain			Binding of cytophilic IgG	(1)
		Induction of cytotoxicity by MAF; cytotoxic effect		(5)
Pronase			Binding of antigen-antibody complexes	(2)
	Inhibition of lymphoblastogenesis			(13)

Table 21. Contd.

Function tested		Reference
Enzyme	Decreased Unchanged Increased	
Pronase (contd.)		
Induction of cytotoxicity by MAF	Cytotoxic effect	Binding of cyto-philic IgG
		(1)
		(5)
	Chemotaxis towards casein	
		(12)
<u>Glycolytic enzymes:</u>		
Neuraminidase		
	Binding of <u>Myco-plasma pulmonis</u>	(4)
	Inhibition of lymphoblastogenesis	(13)
	Binding of cyto-philic IgG	(1)
	Binding of and response to MIF	(8)
	Chemotaxis towards casein	(12)
	Phagocytosis of negatively charged microspheres	(14)

Table 21. Contd.

Enzyme	Function tested			Reference
	Decreased	Unchanged	Increased	
β -Galactosidase		Inhibition of lymphoblastogenesis		(13)
α -L-Fucosidase	Response to MIF	Chemotaxis towards casein		(15) (12)

References:

- (1) Davey & Asherson, 1967.
- (2) Arend & Mannik, 1973.
- (3) Wilkinson, 1975.
- (4) Jones, 1975.
- (5) Dy et al., 1976.
- (6) Huber et al., 1968.
- (7) Ley & Nussenzweig, 1969a.
- (8) Leu et al., 1972.
- (9) Unkeless, 1977.
- (10) Rabinovitch, 1967a.
- (11) Czop et al., 1978.
- (12) Wilkinson, 1976a.
- (13) Baird & Kaplan, 1977.
- (14) Weiss et al., 1966.
- (15) Remold, 1973.

1.3 Mechanisms of binding of non-opsonized bacteria to macrophages.

1.3.1. Electrostatic effects. The experiments reported here did not provide much direct information on the mechanisms involved in the binding of non-opsonized bacteria to macrophages. Electrical charge effects may play a role in the initial balancing of attraction and repulsion. This was indicated by the enhancing effect of neuraminidase treatment on the binding of C. parvum as was explained in the preceding chapter. It does not seem likely, however, that binding itself was primarily affected by electrical forces with reference to the highly specific patterns of inhibition obtained with monosaccharides (see above).

1.3.2. Cellular motility. The binding observed at 4°C was entirely passive and did not involve cellular motility or metabolism as shown by the unimpaired binding of C. parvum to macrophages in the presence of cytochalasin B or following fixation with glutaraldehyde. Cellular motility appeared to contribute significantly to the increased binding observed with increases in temperature to 20°C or 37°C (see Tables 2 & 3 in the "Results") and was clearly responsible for changed patterns of binding at the higher temperatures involving the entire cell surface. Phagocytosis also required cellular motility as expected. The temperature dependence of phagocytosis

observed here appeared to agree well with the results of Rabinovitch (1967b) using normal mouse peritoneal macrophages and glutaraldehyde-fixed horse erythrocytes.

Binding of bacteria at 4°C and initial binding at 20°C was restricted to the circumference of the glass-adherent macrophages. This might be caused by a purely physical effect, that would make it most likely for the bacteria to become lodged in the corner between the cell surface and the glass. Such preferential binding or trapping of particles at the circumference of surface-attached phagocytes has been observed before and was thought to stimulate phagocytosis, a process called "surface phagocytosis" (Foley *et al.*, 1959). Another, perhaps more likely, explanation in the light of present knowledge of plasma membrane structure and motility is that surface components are unevenly distributed on cells that are adhering to and spreading on a surface with intense ruffling at the protruding edges (see "Introduction", 5.3; 7.1.4.). At higher temperatures the presence of particles in the medium may stimulate ruffling over the exposed surface thus accounting for the differences in morphology of binding.

1.3.3. Requirement for Ca^{++} - and Mg^{++} -ions.

It was shown that binding of C. parvum to macrophages at 4°C depend on the presence of both Ca^{++} - and Mg^{++} -ions whilst at room temperature Mg^{++} -ions alone were partially effective in

promoting binding and phagocytosis. A requirement for these divalent cations for binding and phagocytosis is in general agreement with the findings of other workers. Stessel (1973) has shown that 1.0-1.5 mM concentrations of Ca^{++} or Mg^{++} stimulate optimal phagocytosis of unopsonized paraffin oil particles to alveolar macrophages. No phagocytosis occurred in the absence of these cations; Mg^{++} was somewhat more effective than Ca^{++} but their effects were additive. Phagocytosis of starch and latex particles by polymorphonuclear leucocytes in the absence of serum was also dependent on Ca^{++} or Mg^{++} at an optimal concentration of 1 mM (Wilkins & Bangham, 1964). Rabinovitch (1967b) found that either Ca^{++} or Mg^{++} were required for phagocytosis, attachment alone was however not affected by the presence of 5 mM EDTA. The spreading of macrophages on a surface in vitro is induced by Mg^{++} -ions but not Ca^{++} -ions (Rabinovitch & De Stefano, 1973). This process is likely to involve both adherence and cellular motility. The adherence of polymorphonuclear leucocytes to a surface has also been found to require Mg^{++} but not Ca^{++} (Bryant, 1969). These studies tend to favour a somewhat more important role for Mg^{++} than Ca^{++} . It is worth noting that in most of these cases processes involving cellular motility were being investigated.

1.3.4. Biological effects of Ca^{++} - and Mg^{++} -ions.

Calcium and Magnesium ions are well known to have several biological activities some of which may be of importance in the system studied here. Both ions catalyse the action of many enzymes (Williams, 1970), they bind to a variety of biological substances of protein, lipid or carbohydrate nature (Williams, 1970; Prestipino *et al.*, 1974; Rao *et al.*, 1974; Hayashi & Kataguri, 1974). Calcium, in particular, influences the structure of biological material and changes in the concentration of Ca^{++} can for example induce changes in cell morphology (Pinteric *et al.*, 1975) and fluidity and permeability of phospholipid bilayers (Prestipino *et al.*, 1974; Schnepel *et al.*, 1974). Divalent cations, particularly Ca^{++} , Mg^{++} and Mn^{++} are often found to be required for the specific interaction of lectins with saccharides (Sharon & Lis, 1972; Agrawal & Goldstein, 1968; Pauleva *et al.*, 1971; Marchalonis & Edelman, 1968). It is well known that many cellular adhesion phenomena require Ca^{++} (see "Introduction", 8.1.9., 8.3.9.); in some situations Mg^{++} may be more effective than Ca^{++} (Curtis, 1973). Contractile proteins depend on Ca^{++} for their function (see "Introduction", 7.1.6., 13.1.2.) and actomyosin ATP-ase requires Mg^{++} (see Mountcastle, 1974). The ATP-ase activity of macrophage myosin depends on Mg^{++} and Mg^{++} also accelerates the contraction of a gel formed from extracted macrophage contractile proteins (Stossel & Hartwig, 1976).

1.3.5. The role of Ca^{++} and Mg^{++} in the binding of non-opsionized bacteria to macrophages at 4°C . The dependence of binding of non-opsionized bacteria to macrophages at 4°C on Ca^{++} and Mg^{++} observed here is most likely to be directly related to the mechanism of passive adherence although structural effects on plasma membrane components might also be a possibility. An action on enzymes and contractile elements could be involved at the higher temperatures. The possible effects of these divalent cations in cellular adhesion reactions include a bridging function between surface components either directly or via an intermediate bridging molecule, or a reduction in electrostatic repulsion (see "Introduction", 8.1.9.). Wilkins & Bangham (1964) showed that the surface charge of the phagocytes, as tested by electrophoretic mobility, was affected by Ca^{++} or Mg^{++} only at concentrations higher than those optimal for phagocytosis. The divalent cations were required for the phagocytosis of negatively charged (latex) as well as uncharged (starch) particles. These authors, therefore, concluded that the effects of Ca^{++} and Mg^{++} on phagocytosis could not be explained in terms of changes in electrostatic repulsion in a system governed by the forces described by the "DLVO" theory of colloid stability (see "Introduction", 8.1.6.).

The observation by Cook & Bugg (1975) that Ca^{++} can form

a bridge between uncharged sugars appears to be particularly relevant to the present results. These authors have shown that Ca^{++} binds readily to uncharged carbohydrates via their hydroxyl groups and that even in anionic carbohydrates Ca^{++} binding occurs to the hydroxyl groups as well as the carboxylate groups. The hydroxyl groups appear to replace some of the water molecules in the hydration shell of the Ca^{++} -ion. There is a strict requirement for the appropriate geometrical arrangement implying that Ca^{++} cross-linking of carbohydrates could form the basis of a stereo-specific recognition mechanism. Calcium-bridging of this type has been demonstrated between two fucose molecules (Cook & Bugg, 1975) and between two lactose molecules where the glucose of one of the two lactose molecules and the galactose of the other are bound to the Ca^{++} -ion (Bugg, 1973). The binding of three galactose molecules to one Ca^{++} -ion involving five different hydroxyl groups, one or two from each of the galactose molecules has also been observed (Cook & Bugg, 1973). It is attractive to speculate that stereo-specific Ca^{++} -bridging between carbohydrates of this kind was involved in the binding of unopsonized bacteria to macrophages described here. It must be noted, however, that at 4°C both Ca^{++} - and Mg^{++} -ions were required. One possible explanation is that the presence of Mg^{++} increased the availability of free Ca^{++} -ions in the phosphate-buffered

medium. Alternatively Mg^{++} may have had necessary additional effects such as reduction of repulsive charge forces. Bridging between carbohydrates by Mg^{++} has not been described. It is, however, known that Mg^{++} binds more strongly than Ca^{++} to weakly acidic groups or neutral groups such as amines and alcohols in biological systems (Williams, 1970).

1.3.6. The role of Mg^{++} -ions in the binding and phagocytosis of bacteria at 20°C and 37°C. The limited stimulatory effect of Mg^{++} alone at the higher temperatures can not be explained solely on the basis of the passive binding reaction. As described above Mg^{++} -ions are of importance in macrophage motility and spreading. Most glycosyltransferases require divalent cations for their function (see preceding chapter). The active cation has often been found to be Mn^{++} but can in some cases be Mg^{++} whilst Ca^{++} is often inhibitory (Roseman, 1970). It has already been mentioned in the preceding chapter that macrophages are likely to contain glycosyltransferases in their plasma membrane (Canonico et al., 1978). The Mg^{++} -ions could thus have aided attachment at room temperature by stimulating the movement of plasma membrane components thus increasing the chance of a surface binding site configuration suitable for binding. Alternatively or in addition the action of glycosyltransferases may contribute to the establishment of a link between the macrophage surface

and the bacterial cell wall carbohydrates.

1.3.7. Concluding remarks. On the basis of the evidence discussed above it may be envisaged that the attachment of non-opsonized bacteria to macrophages involves first a passive adherence between surface carbohydrates that relies on bridging by divalent cations and a subsequent strengthening of this bond by the action of glycosyltransferases if the reaction occurs at room temperature or 37°C.

1.4 Biological significance of carbohydrate-mediated non-specific recognition of bacteria by macrophages.

1.4.1. Carbohydrate-mediated recognition in invertebrates. The form of carbohydrate-mediated recognition of non-opsonized bacteria by phagocytic cells described here may represent a primitive recognition mechanism that could operate in species that do not possess specific antibodies. In contrast to the complex and flexible surface architecture of eukaryotic cells with many different proteins and glycoproteins embedded randomly in a phospholipid bilayer membrane, prokaryotic cells display more regular, repeating patterns, composed to a large extent of carbohydrates, on their surfaces (see Ghuyssen, 1977). It therefore seems logical that recognition of prokaryotic cells by nucleated cells should be mediated by carbohydrates.

There are numerous recorded examples of carbohydrate-mediated recognition in invertebrates. Species-specific aggregation of sponge cells may be mediated by carbohydrate-specific aggregation factors (Turner & Burger, 1973). The simultaneous secretion of lectins and increased expression of galactose residues on the surface appears to enable slime moulds to change from the non-cohesive to the cohesive state (Reitherman et al., 1975). Many invertebrates including snails, molluscs, lobsters, crabs and others, contain in their tissue

fluids substances that agglutinate erythrocytes and sometimes also bacteria although the reaction with bacteria has not been as extensively studied (Uhlenbruck, 1974; Jenkin, 1976). Some of these agglutinins have been shown to have carbohydrate-specificities and can therefore be classified as lectins. Thus haemagglutinins from horseshoe crabs (Limulus polyphemus) and coconut crab (Birgus latro) are specific for N-acetylneuraminic acid (Cohen, cited by Uhlenbruck, 1974) and one of two agglutinins identified in the haemolymph of the lobster (Homarus americanus) shows the same specificity whilst the other lobster agglutinin is specific for N-acetylgalactosamine (Hall, cited by Uhlenbruck, 1974). The haemagglutinin from the haemolymph of snails (Helix pomatia) reacts with both N-acetylgalactosamine and N-acetylglucosamine (Renwranztz, cited by Uhlenbruck, 1974). "Tridacnins", the agglutinins of bivalve clams are specific for β -galactosyl residues (Uhlenbruck, 1977). There is some evidence to indicate that these agglutinins can act as opsonins. This is implied by the immunological cross-reactivity of the haemagglutinins and bacterial opsonins of crayfish (Jenkin, 1976) and phagocytosis of human erythrocytes by lobster haemocytes only in the presence of the N-acetylneuraminic acid-reactive haemagglutinin (Hall, cited by Uhlenbruck, 1974). The phagocytosis by cockroach haemocytes of unopsonized erythrocytes and bacteria (Scott, 1971; Anderson et al., 1973) has not been studied with

regard to chemical specificity but would seem likely to involve recognition of surface carbohydrates. The cells producing the invertebrate agglutinins and opsonins have not been identified (see Jenkin, 1976) and it is thus not known whether these substances might to some extent be regarded as detached surface receptors of phagocytic cells.

1.4.2. Carbohydrate-mediated recognition in mammals. Carbohydrate-mediated recognition appears thus to be important in the maintenance of integrity and distinction between self and non-self by invertebrates. There is now evidence to suggest that mammalian species have to some extent retained this ability to recognize carbohydrates. Ashwell and coworkers have studied in detail the galactose-specific binding by rabbit hepatocytes of asialated glycoproteins (see Ashwell & Morrell, 1974). This binding requires the presence of Ca^{++} -ions. The glycoprotein receptor has been isolated from hepatocyte plasma membranes and found to be composed of two glycopeptide subunits (Kawasaki & Ashwell, 1976a & b). The carbohydrate portion contains sialic acid, galactose, N-acetylglucosamine and mannose and the sialic acid appears to be required for the binding of asialoglycoproteins (Kawasaki & Ashwell, 1976b; Ashwell & Morrell, 1974). Galactose-binding lectins have been isolated from calf heart and lung (De Waard et al., 1976). Stahl et al. (1978) have recently reported the binding to rat alveolar macrophages of

glycoproteins containing mannose, N-acetylglucosamine or glucose in the terminal position. Glycoproteins with terminal galactose residues were not bound. Binding was reduced by 70% following exposure of the cells to trypsin (0.1 mg/ml) for 15 minutes. Kolb & Kolb-Bachofen (1978) have investigated the passive binding of neuraminidase-treated rat erythrocytes to rat Kupffer cells and found that this is inhibited by N-acetylgalactosamine and galactose but not glucose or mannose. The studies of Stahl et al. and Kolb & Kolb-Bachofen agree quite well with the findings of the present work on the binding of non-opsonized bacteria to mouse peritoneal macrophages.

1.4.3. The role of relative hydrophobicity in non-specific recognition by phagocytes. The factors implicated by previous authors in non-specific recognition by phagocytes include relative hydrophobicity and surface electric charge (see "Introduction" 8.3.6., 8.3.7., 8.3.8.). It has also been suggested that recognition of bacteria could be mediated by receptors for bacterial formylmethionyl peptides (see "Introduction", 8.3.6.).

Van Oss and co-workers have used contact angle measurements as an indication of hydrophobicity of bacteria and cells and have come to the conclusion that non-opsonized bacteria are phagocytosed only if they give a contact angle greater than the phagocytes, indicating a higher degree of hydrophobicity

(van Oss & Gillman, 1972a; van Oss, 1978; see also "Introduction", 8.3.5.). The lower degree of binding to polymorphonuclear leucocytes and HeLa cells of smooth strains of S. typhimurium as compared with rough strains has also been correlated with greater hydrophobicity as estimated by separation in polar and non-polar medium (Stendahl & Edebo, 1972; Stendahl et al., 1973; Kihlström & Edebo, 1976). According to Wilkinson (1976a) substances that have chemotactic activity are usually relatively hydrophobic. A chemotactic effect may involve more than simple surface recognition and interactions between the hydrophobic molecules and the lipid bilayer of the plasma membrane may contribute to the activity of these substances. The only indication of involvement of these surface properties in the present work was the failure of the capsulated (and therefore presumably relatively hydrophobic) Str. pneumoniae to bind to macrophages to a significant degree. There were, however, no differences in the binding ability of the capsulated and uncapsulated strains of K. aerogenes.

1.4.4. Electrostatic forces in non-specific recognition by phagocytes and bacterial adherence. The influence of surface electric charge was already mentioned and it was noted that a reduction in the negative surface charge of phagocytes can enhance the binding and phagocytosis of negatively charged particles (see two preceding chapters).

Nogura et al. (1977) treated rat macrophages and glutaraldehyde-treated sheep erythrocytes with the polycation protamine. This stimulated phagocytosis of the erythrocytes. In contrast Knyszynski et al. (1978) have recently concluded that differences between stimulated and unstimulated mouse macrophages in their ability to phagocytose young and old mouse erythrocytes do not correlate with differences in surface charge density as measured by labelling with cationic ferritin. Electrostatic forces may be important in the adherence of bacteria to dead or living surfaces. Thus Marshall et al. (1971) showed, by changing the ionic strength of the medium, that the initial reversible sorbtion of two marine bacteria to glass is determined by the balance between electrostatic repulsion and van der Waals attraction. The stage of reversible sorbtion when the bacteria could be removed by washing was in the case of a strain of Pseudomonas followed by the second stage of irreversible attachment which involved the production of polymeric fibrils. Similarly Rutter & Abbott (1978) have recently studied the adherence of Str. salivarius and Str. mitior to rotating glass or polystyrene discs. The balance between electrostatic and van der Waals forces was shown to be important but in addition a "fuzzy layer" around Str. salivarius, which could be removed by trypsin, contributed significantly to the adsorbtion of this organism. Blocking of carboxyl groups on Neisseria gonorrhoeae

which results in reduction of negative surface charge enhances the binding of these bacteria to human amnion cells. The presence of pili has no effect on the adherence of these modified bacteria whilst pili stimulate the adherence of normal, negatively charged bacteria. These findings indicate that the pili may help to overcome the initial electrostatic repulsive barrier between cells and bacteria (Heckels et al., 1976). The results discussed above imply that the physical forces of electrical repulsion and van der Waals attraction may govern the probability of close contacts between bacteria and dead or living surfaces but the final stage of adherence may depend on the chemistry of the bacterial surface structures.

1.4.5. Relative importance of physico-chemical factors in non-specific recognition by phagocytes. As discussed in the preceding chapter the enhancing effect of neuraminidase-treatment on the binding of bacteria to macrophages can be explained by a reduction in the negative surface charge of the macrophages thus suggesting that electrostatic forces normally exert a repulsive influence.

It appears from the present work that non-specific phagocytic recognition is promoted by favourable physico-chemical conditions but that biochemically more specific interactions involving surface carbohydrates form the basis of the recognition of non-opsonized bacteria. This implies that the recognition

mechanism is specific for biochemical characteristics shared by most bacteria but does not select between different species or strains of bacteria in the manner of specific antibodies.

1.4.6. Recognition of bacterial formylmethionyl peptides by phagocytes. Bacterial formylmethionyl peptides have been shown to be chemotactic for polymorphonuclear leucocytes and macrophages (Schiffmann et al., 1975a; Williams et al., 1977). These peptides are produced during protein synthesis in prokaryotic cells (Schiffmann et al., 1975a) but do not appear to be exposed on their surface (see Ghuyssen, 1977). They may therefore attract phagocytic cells to the site of an infection but surface attachment of bacteria is likely to involve different mechanisms. It was noted in the "Introduction" (9.1.4.) that factors mediating macrophage chemotaxis and macrophage adherence may be different in nature even if they have the same or related origin.

1.4.7. Inhibition of phagocytosis by polysaccharides and glycoproteins. There are some indications from other work that carbohydrates may be involved in the recognition by phagocytes of non-opsonized particles. The study of Kolb & Kolb-Bachofen (1978) on the role of N-acetylgalactosamine in the recognition of modified erythrocytes by Kupffer cells was mentioned above. Kozel & Mastroianni (1976) showed that

capsular polysaccharide from Cryptococcus neoformans inhibited the attachment phase preceding phagocytosis of a non-encapsulated strain of this organism by normal mouse macrophages. Organisms of the encapsulated strain were bound and phagocytosed only in very low numbers. The presence of 10% calf serum stimulated the rate of attachment of the non-encapsulated strain at 4°C but the degree of binding after 2 hours was the same with or without serum. Both the rate and degree of phagocytosis at 37°C were greatly enhanced by the presence of serum. As the inhibition experiments with the capsular polysaccharide were only performed in the presence of serum it can not be determined whether the polysaccharide affected the function of serum opsonins or the direct binding of the organisms to the macrophages. Specific antibody against a conjugate of the polysaccharide and albumin was found to enhance phagocytosis of an encapsulated strain although attachment in the cold was not markedly increased. Similarly Dri et al. (1976) have shown that a polysaccharide-containing extract from strains of E. coli that are resistant to phagocytosis by guinea pig polymorphonuclear leucocytes and macrophages could inhibit the phagocytosis of other strains of E. coli or polysaccharide-free E. coli that are normally susceptible to phagocytosis. The authors did not state whether the medium contained serum. In the experiments of both Dri et al. and Kozel & Mastroianni the inhibitory polysaccharides were added to the incubation mixture

of micro-organisms and phagocytes and could therefore have acted at either the phagocyte surface or the surface of the micro-organisms or both. Dri et al. (1976) showed that the polysaccharide could be adsorbed by the E. coli and concluded that the substance modified the surface properties of the bacteria. The findings of Bole and coworkers were already briefly mentioned in the "Introduction" (8.3.10.). These workers found that a glycoprotein, containing glucose, mannose, galactose, glucosamine and neuraminic acid, that can be isolated from chronic granulomas inhibited the phagocytosis of St. aureus by guinea pig macrophages but had no effect on phagocytosis by polymorphonuclear leucocytes (Bole et al., 1975). The glycoprotein was slightly inhibitory when preincubated with either the bacteria or the macrophages and appeared therefore to be able to bind to both. Immunofluorescence showed that the material occurred on the surface of and/or inside macrophages in the granulomas (Bole & Wright, 1976). Friedberg & Shilo (1965) reported that a systemic injection of several different polysaccharides 4 to 6 hours before an intraperitoneal inoculation with an avirulent strain of Pasteurella pestis enhanced the infectivity of these organisms in mice. Peritoneal lavage before inoculation also led to highly increased susceptibility of the animals. It is tempting to speculate that the effects observed were caused by the blocking of recognition mechanisms

of phagocytes by the injected polysaccharides or removal of the local macrophages in the case of lavage.

1.4.8. Long carbohydrate chains and escape from phagocytosis. Long carbohydrate chains have been associated with high infectivity and effective escape from phagocytosis in vivo and in vitro by strains of E. coli (Medearis et al., 1968) and Salmonellae (Nakano & Saito, 1968; Friedberg & Shilo, 1970). From the present study this would seem to be a paradox as organisms with more surface sugars might be expected to bind more readily to phagocytes. One possibility is that the phagocytic effort results in the shedding of some of the surface carbohydrates from the bacterium leaving it free to detach. It has been observed that some bacteria release their surface carbohydrates quite readily whilst remaining viable (Dr. Ian Sutherland, personal communication). It may be speculated that such released carbohydrates could possibly interfere with carbohydrate-mediated recognition by phagocytes. Although escape from phagocytosis has been recognised as a virulence factor for a long time (see Dubos, 1945) the mechanisms involved still remain to be clarified.

1.4.9. Non-specific recognition by macrophages in the induction of the specific immune response. Mammalian macrophages are frequently involved in recognition reactions without the intervention of specific antibodies. Antibody-

independent recognition by macrophages is likely to be important in the elimination of a large proportion of a bacterial inoculum in a non-immune animal (Friedberg & Shilo, 1965; Medearis et al., 1968) and has to occur in the initial focussing of antigen by macrophages in the induction of the specific immune response. Recognition in these cases could to some extent involve non-specific opsonins such as complement but these may not be readily available in some anatomical situations such as the lung (Newhouse et al., 1976). It is thus conceivable that the carbohydrate-mediated recognition mechanism described here may play an important part in the initial binding of bacteria and other carbohydrate-containing antigens to macrophages preceding their presentation to lymphocytes.

1.4.10. Impaired immune function in diabetes mellitus. The minimum effective concentration of glucose (10 mM) that inhibited binding of bacteria to macrophages is somewhat higher than that normally found in body fluids but is well within the range of blood glucose levels frequently encountered in diabetes mellitus. It is well known that diabetes is associated with an increased susceptibility to infections. Cell-mediated immune responses have been found to be impaired in hyperglycaemic diabetic humans and animals (MacCuish et al., 1974; Ptak et al., 1975). Antibody

responses have sometimes been found to be normal (Dolkart et al., 1971) although a recent study showed decreased responses to T-cell-dependent and T-cell-independent antigens in alloxan diabetic mice with high blood glucose levels (≥ 20 mM) (Ptak et al., 1977). Cell transfer experiments showed that lymphocytes could not get optimally sensitized in diabetic animals as they produced subnormal secondary responses when transferred into normal animals; conversely normal sensitized lymphocytes gave decreased responses when transferred into diabetic animals (Ptak et al., 1975 & 1977). This indicates that the microenvironment of diabetic animals is suppressive for primary and secondary immune responses. It may be speculated that one of the contributing factors is interference by the high glucose concentration with the initial uptake of antigen by macrophages and therefore less efficient subsequent presentation to lymphocytes.

1.4.11. Carbohydrate mediated recognition of rapidly dividing cells by macrophages. Recognition of surface carbohydrates might be important in the interaction of macrophages with rapidly dividing cells (see "Introduction", 8.3.3.; 12.5.3.). It was already mentioned in the "Introduction" (7.1.8.) that one of the differences between malignant cells or normal mitotic cells and normal non-dividing cells is an altered expression of surface carbohydrates as reflected in agglutinability by lectins. Work has now been carried out

in this laboratory showing that the binding of mouse macrophages to syngeneic fibrosarcoma and mammary carcinoma cells as well as rapidly growing embryonic fibroblasts can be inhibited by a range of monosaccharides and is affected by pre-treatment of the macrophages with enzymes and periodate much in the same way as described here for the binding of bacteria (Weir et al., 1979). There is thus evidence to suggest that the carbohydrate-mediated recognition mechanism extends to rapidly dividing cells. The possible function of macrophages in limiting malignant growth and regulating normal cell proliferation of e.g. lymphocytes was described in the "Introduction" (12.5.5.; 11.1.8.).

2. MECHANISMS OF MACROPHAGE ACTIVATION.

2.1 Stimulation of phosphatidylinositol turnover in macrophages by activating agents but not phagocytic stimuli.

2.1.1. Dissociation of the effects of activating and purely phagocytic stimuli. The results presented here showed that the macrophage activating agents bacterial lipopolysaccharide (endotoxin) and C. parvum induced an increase in the uptake of radiolabelled myo-inositol by macrophages whilst ingestion of particles that do not cause activation (latex, carbon, St. albus) did not stimulate the uptake. Furthermore C. parvum had a stimulatory effect only in the presence of T-cells or if the macrophages had been previously stimulated by an infection in the mice. This is in accordance with the reported dependence of macrophage activation by C. parvum in vitro on the presence of T-cells or previous stimulation in vivo (Christie & Bomford, 1975; Ghaffar & Cullen, 1976; Šljivić & Watson, 1977). Both C. parvum with T-cells and endotoxin were shown to stimulate the synthesis and secretion of lysosomal enzymes by macrophages after 24 and 48 hours whilst C. parvum without T-cells and latex particles had no such effect. Treatment of macrophages with endotoxin also resulted in enhanced bacteriostatic activity against Listeria monocytogenes after 24 hours. As all the test particles were phagocytosed and the presence or absence of T-cells had no effect on the

degree of uptake of C. parvum these findings demonstrate an early biochemical response of macrophages to macrophage-activating stimuli that is not linked to the process of phagocytosis itself.

A similar dissociation of phagocytosis and early biochemical responses to ingested material was recently reported by Schnyder & Baggiolini (1978). These authors showed that formalin-treated sheep erythrocytes and zymosan particles triggered both an early stimulation of glucose oxidation via the hexose monophosphate shunt pathway and, over several days in culture, a release of β -glucuronidase and plasminogen activator. Latex particles caused no metabolic stimulation. All three particles were ingested. The biochemical changes that occur during macrophage activation appear thus to be mediated by pathways separate from those operating during the engulfment of particles.

2.1.2. Increased turnover of phosphatidylinositol by macrophages. The measured increase in uptake of radio-labelled myo-inositol is interpreted to indicate an increase in the turnover of macrophage cell membrane phosphatidylinositol. Virtually all the myo-(2-³H)-inositol that was taken up was shown to be incorporated into phosphatidylinositol. With reference to current knowledge of the metabolism of phosphatidylinositol (see "Introduction", 13.1.6.) this can be taken to reflect an increased rate of turnover of the phosphorylinositol headgroup of phosphatidylinositol. Although increased de novo synthesis

was not formally excluded in the present experiments this is not likely to have contributed greatly to the observed increase in uptake of labelled myo-inositol. Resynthesis of plasma membrane phospholipids is known to take place in macrophages following ingestion of latex particles but this only starts after a lag period of about 6 hours (Werb & Cohn, 1972) and phagocytosis alone had no effect on the uptake of inositol into phosphatidylinositol in the present study. Sbarra & Karnovsky (1960) also found that the uptake of labelled phosphorus into the lipids of phagocytosing polymorphonuclear leucocytes was more marked than the incorporation of labelled acetate, indicating a higher rate of turnover of the headgroups of phospholipids as compared with the rate of resynthesis. The labelled phosphorus was found to be mainly incorporated into lipid fractions containing phosphatidic acid, phosphatidylserine and phosphatidylinositol (Karnovsky & Wallach, 1961). There is good evidence that enhanced incorporation of myo-inositol or phosphorus into phosphatidylinositol occurs without an increase in de novo synthesis in non-phagocytic cells, e.g. lymphocytes (Fisher & Mueller, 1971; Michell, 1975).

No attempt was made in the present study to investigate which subcellular fractions contributed to the increased uptake of myo-inositol. By looking at the model of enhanced phosphatidylinositol turnover shown in 13.1.6. in the "Introduction" it can be seen that the effect was most likely initiated by a

contact with (a) surface component(s) in the plasma membrane but that the increased incorporation of inositol took place on the endoplasmic reticulum (see also Hokin, 1968).

Phosphatidylinositol exchange proteins will then serve to transport phosphatidylinositol between the endoplasmic reticulum and the plasma membrane.

The increased myo-inositol uptake observed was shown not to be caused by increased adherence of the macrophages to the culture tube or reduced cell death. The assay system was also not very sensitive to some fluctuations in the concentrations of unlabelled inositol. Splenic lymphocytes exhibited a proliferative response to C. parvum and when they were added with C. parvum to the macrophages the uptake of myo-inositol by these lymphocytes may thus also have been stimulated. Increased phosphatidylinositol turnover is known to occur in mitogen-stimulated T-cells (Fisher & Mueller, 1971; Maino et al., 1975) and appears also to be stimulated in B-cells by treatment with anti-immunoglobulin (Maino et al., 1975) although B-cells may not respond to mitogens in this way (Betel et al., 1974). The splenic lymphocytes were effectively washed away at the end of the incubation period and did therefore not contribute to the measured radioactivity. It might be speculated that the presence of lymphocytes had a more indirect effect by consuming unlabelled inositol before the addition of the labelled substance but supernatants of cultures of spleen cells and C. parvum kept

under comparable conditions for the same length of time would then have been expected to have a similar effect. This was not the case.

The confusing results obtained with supernatants from longer term spleen cell cultures and effects of storage on these supernatants were not investigated further and remain unexplained. Measurements of fluctuations in the concentrations of myo-inositol and phospholipids along with investigations of the chemical nature of interfering substance(s) by e.g. heating, dialysis or chemical treatment might provide some insight but this was beyond the scope of the present study.

It can be concluded from the above that the measured increase in the uptake of myo-inositol is likely to have reflected an increase in the turnover of membrane phosphatidyl-inositol in the macrophages initiated by a stimulus acting at the cell surface.

2.1.3. Increased phosphatidylinositol turnover not directly involved in endocytosis or exocytosis. The present observations contrast with the earlier findings of Karnovsky and co-workers that phagocytosis as well as exposure to the surface-active agents endotoxin, digitonin and deoxycholate stimulates the uptake of radiolabelled phosphorus into phosphatidylinositol in both polymorphonuclear leucocytes and macrophages (Oren et al., 1963; Graham et al., 1967; Karnovsky et al., 1970). In their experiments casein-induced guinea pig

macrophages were used although it was stated that similar results were obtained using normal mouse peritoneal macrophages but these results were not shown (Graham et al., 1967). It is well known that macrophages that have been stimulated in vivo respond in a different way to further stimulation in vitro as compared with normal macrophages (see "Introduction", 12.1.2., 12.2.3.). It is perhaps also noteworthy that the test particle used by Karnovsky and co-workers in the studies just cited was starch. Goldman & Hogg (1977) have recently described that peritoneal macrophages elicited in vivo with starch had cytotoxic effects against transformed or non-transformed virus-infected cells and appeared thus to be activated.

Increased phosphatidylinositol turnover is not involved in the process of phagocytosis according to the present study. Phosphatidylinositol is a very minor constituent of the macrophage plasma membrane, only accounting for approximately 6% of the total membrane phospholipids (Mason et al., 1972) and yet the metabolism of this phospholipid was more markedly stimulated than that of other phospholipids by phagocytosis in the studies of Karnovsky and co-workers. It is therefore likely that their results were indicating a more specific metabolic response to the encounter with particles rather than merely the effect of endocytosis on the overall turnover of cellular membranes.

In the early work of Hokin and co-workers the suggestion was made that the specific increase in phosphatidylinositol metabolism in secretory cells was connected to the synthesis of cellular membranes or molecular transport between cellular membranes in the process of secretion (Hokin & Hokin, 1965; Hokin, 1968). It is now clear that increased phosphatidylinositol turnover occurs in many cells in response to stimuli that do not elicit endocytosis or exocytosis, for example the stimulation of proliferation of lymphocytes (Maino et al., 1975) and L-cells (Shearer & Crouch, 1977) and stimulation of postsynaptic neurones in sympathetic ganglia (see Michell, 1975). As mentioned in the "Introduction" (13.1.6.) there are now suggestions that increased turnover of phosphatidylinositol is involved in the opening up of Ca^{++} gates in the plasma membrane in the signal transmission across the membrane. This hypothesis gains support from recent experiments by Fain & Berridge (1979) on isolated blowfly salivary glands. These authors have shown that stimulation of fluid secretion by 5-hydroxytryptamine was reduced in the absence of Ca^{++} but breakdown of prelabelled phosphatidylinositol was still stimulated. 5-hydroxytryptamine increases the permeability of the gland epithelium to Ca^{++} and this effect was shown to be directly related to the cellular concentration of phosphatidylinositol (Berridge & Fain, 1979).

2.1.4. Link between cyclic GMP, calcium and increased turnover of phosphatidylinositol. The link between mediation by cyclic GMP, Ca^{++} and increased phosphatidylinositol turnover of cellular stimulation was outlined in the "Introduction", 13.1.6.. The present findings on enhanced phosphatidylinositol turnover by macrophages in response to activating stimuli can therefore be said to be in agreement with previous indications that macrophage activation might be mediated by cyclic GMP or divalent cations (see "Introduction", 13.2.1., 13.1.1.).

2.2 Time course of stimulated phosphatidylinositol turnover by macrophages as related to the development of the activated state.

2.2.1. Time course of stimulation and activation of macrophages in vitro. In order to get a clearer picture of the sequence of events during macrophage stimulation and activation in vitro Table 22 (p.356) was compiled from some of the data available in the literature. Three different stages may be distinguished. During the first few hours (up to approximately 6 hours after applying a stimulus) responses involving cellular motility occur, viz. adherence to surfaces, phagocytosis, chemotaxis as well as migration inhibition. Stimulation of respiration, increased glucose oxidation via the hexose monophosphate shunt as well as release of prostaglandins is also noted. The next stage extends over the following 24-36 hours and is mainly characterized by the release of pre-formed substances such as lysosomal enzymes and lymphocyte activating factor. The final stage involves increased synthesis as well as secretion of lysosomal enzymes, synthesis and secretion of neutral proteinases and full expression of cytotoxic activity and microbicidal effects on intracellular parasites. These latter two major characteristics of activated macrophages take as long as 48-72 hours to develop during activation in vitro. It can be seen that the time course varies somewhat with different experimental systems depending on the activating agent and previous stimulation of the macrophages but the order of events appears to be fairly constant.

The changes of the first stage occur frequently without leading on to the second or third stages and may, therefore, be controlled by separate mechanisms, as suggested in the preceding chapter. Mediation of motility responses may involve electrophysiological activity as described in the "Introduction" (9.1.3., 9.2.3.). The second stage is intermediate in character; it may not be followed by the third stage although this is often the case. Secretion alone may be noted quite early on and might depend mainly on the stimulation of fusion of cellular membranes and increased activity of cytoskeletal elements which are stimulated during the first few hours. A stimulant that causes prolonged secretion such as Streptococcal cell walls (Davies et al., 1974) and lymphokines (Pantalone & Page, 1977) often leads eventually to increased synthesis and it seems possible that different control mechanisms take over when this occurs. From the time course of activation it appears that the mediating events leading to the increased synthetic activity of the macrophages with full cytotoxic and antibacterial capacity take place within the first 24 hours.

2.2.2. Possible transmission of activating signal by increased phosphatidylinositol turnover. The time course of increased phosphatidylinositol turnover in response to endotoxin showed a maximum at 6 hours and was declining at 24 hours. The increase in phosphatidylinositol turnover was shown

to precede the development of enhanced bacteriostatic activity against Listeria as well as secretion and enhanced synthesis of the three lysosomal enzymes measured. Both of these responses were not marked until 24-48 hours after beginning of culture with endotoxin. Stimulation of lysosomal enzyme secretion by C. parvum with T-cells showed the same time course as was observed for endotoxin. These findings together with the close correlation between the ability of a stimulus to induce accelerated turnover of phosphatidylinositol and stimulate bacteriostatic activity and/or lysosomal enzyme secretion indicate a possible involvement of phosphatidylinositol turnover in signal transmission across the macrophage plasma membrane during exposure to an activating agent.

2.2.3. Development of anti-Listerial activity.

It may seem that the anti-Listerial activity was evident in the present study somewhat earlier than is sometimes reported but it must be noted that the macrophages were bacteriostatic against the Listeria but only slightly bactericidal. Endotoxin appears also to be a fairly rapidly acting activating agent (Alexander & Evans, 1971).

2.2.4. Final common pathway of activation. The

question arises whether a final common pathway of macrophage activation exists. It was noted in the "Introduction" (12.4.) that there are some variations in the pattern of functions

induced by different stimuli. Thus stimuli that cause increased production of lysosomal enzymes often have no effect on the production of neutral proteinases and neutral proteinases may be secreted by macrophages that show neither of the two major characteristics of activated macrophages, cytotoxic and antimicrobial activity. It is possible that final adjustments and modulations are governed by the particular plasma membrane site interacting with the stimulant and substances acting as substrates for specific enzymes may induce and maintain their synthesis (Mørland & Mørland, 1978). It is known that cyclic nucleotides may mediate different actions depending on the hormone and type of receptor involved (see "Introduction", 13.1.4.). The two activating agents used in the present study had very similar effects on both the turnover of phosphatidylinositol and the synthesis and secretion of lysosomal enzymes. It would be interesting to compare the effects on phosphatidylinositol turnover of stimuli that produce different final responses.

Table 22. Time course of macrophage stimulation and activation in vitro.

Type of cells Stimulator (References)	Effects observed after:			
	≤ 1 hour	2-6 hours	24 hours	24-48 hours 72 hours
Normal and stimulated macrophages. Particles.	Phagocytosis			
Normal and stimulated macrophages. Surfaces (glass or plastic).	Adherence			
Stimulated guinea pig macrophages. Starch particles. (1)	↑ respiration			
	↑ glucose oxidation via HMPS			
Stimulated guinea pig macrophages. Lymphokines. (2)	↑ turnover of phospholipids			
	↑ glucose oxidation via HMPS			
Normal and stimulated macrophages. C5a, denatured protein, casein etc. (3), (4)	↓ cell permeability			
	Chemotaxis			
	↑ glucose oxidation via HMPS and Krebs ↑ β-glucuronidase activity Cell permeability back to normal			

Table 22. Contd.

Type of cells Stimulator (References)	Effects observed after:			
	≤ 1 hour	2-6 hours	24 hours	24-48 hours 72 hours
Stimulated guinea pig macrophages in suspen- sion culture. Lymphokines. (5)		Migration inhibition		Cytotoxicity
Normal mouse macrophages. Lymphokines. (6)	↑ spreading and granulation	(↑) secretion of β-galactosidase and N-acetyl- -D-glucosaminidase		↑ protein synthesis ↑ synthesis and secretion of collagenase and several lysosomal enzymes ↑ synthesis of IDH and leucine-2- naphthylamidase Release of non- specific macrophage cytotoxic factor
Alloimmune mouse macrophages. Specific alloantigen. (7), (8)		Release of specific macrophage cytotoxin		
Normal mouse macrophages. Streptococcal cell walls, zymosan. (9), (10)		Release of lysosomal enzymes (total con- centration unchanged)		↑ protein synthesis ↑ synthesis of lyso- somal enzymes and IDH and leucine- 2-naphthylamidase

Table 22. Contd.

Type of cells Stimulator (References)	Effects observed after:				
	≤ 1 hour	2-6 hours	24 hours	24-48 hours	72 hours
Normal mouse macrophages. Zymosan. (11)	Production of prostaglandins			Production of prostaglandins	
Normal mouse macrophages. Cyclic GMP. (12)		Release of LAF			
Normal mouse macrophages. Lipopolysaccharide, concanavalin A. (13), (14)			Release of LAF		
Stimulated mouse macrophages. <u>Listeria</u> , latex, <u>BeSO₄</u> . (15)			↑ release of LAF	↑ release of LAF	Release of LAF declining again
Stimulated guinea pig macrophages. Lipopolysaccharide, lymphokines. (16, (17)				Production of collagenase	Production of collagenase

Table 22. Contd.

Type of cells Stimulator (References)	Effects observed after:				
	≤ 1 hour	2-6 hours	24 hours	24-48 hours	72 hours
Stimulated guinea pig macrophages. Immune lymphocytes and antigen. (18)			Bacteriocidal activity against <u>Listeria</u>		
Normal mouse macrophages. Double-stranded RNA, lipopolysaccharide. (19)				Non-specific cytotoxicity	
Normal rabbit alveolar macrophages. Ionophore A23187. (20)			No response		↑adherence ↑acid phosphatase content Bacteriocidal activity against <u>Listeria</u>
Stimulated guinea pig macrophages. Lymphokines. (21), (22)			No response	No response	↓total concentrat- ion of lysosomal enzymes ↑adherence ↑membrane ruffling ↑glucose oxidation ↑phagocytosis

Table 22. Contd.

Type of cells Stimulator (References)	Effect observed after:				↑ number of Fc-receptors
	≤ 1 hour	2-6 hours	24 hours	24-48 hours	
Stimulated, immune guinea pig macrophages. Specific antigen. (23)			No response	No response	↑ glucosamine incorporation
Normal guinea pig macrophages. Foetal calf serum, aged guinea pig serum. (24)					

References:

- (1) Karnovsky et al., 1970.
- (2) Poulter & Turk, 1975a & b.
- (3) Snyderman & Mergenhagen, 1976.
- (4) Wilkinson, 1976b.
- (5) Churchill et al., 1975.
- (6) Pantalone & Page, 1977.
- (7) McIvor & Weiser, 1971.
- (8) Kramer & Granger, 1972.
- (9) Davies et al., 1974.
- (10) Schorlemmer et al., 1977c.

Table 22. Contd.

References: (contd.)

- | | | | |
|------|------------------------------|------|----------------------------|
| (11) | Humes <u>et al.</u> , 1977. | (18) | Simon & Sheagren, 1972. |
| (12) | Diamantstein & Ulmer, 1976. | (19) | Alexander & Evans, 1971. |
| (13) | Gery & Waksman, 1972. | (20) | Hand <u>et al.</u> , 1977. |
| (14) | Gery & Wiener, 1975. | (21) | Remold & Mednis, 1972. |
| (15) | Unanue <u>et al.</u> , 1976. | (22) | David & Remold, 1976. |
| (16) | Wahl <u>et al.</u> , 1974. | (23) | Hammond & Dvorak, 1972. |
| (17) | Wahl <u>et al.</u> , 1975. | (24) | Rhodes, 1975a. |

Abbreviations:

HMS: hexose monophosphate shunt. LDH: lactic dehydrogenase. LAF: Lymphocyte activating factor.

↑: increased, ↓: decreased.

2.3 Direct activating effect of bacterial lipopolysaccharide (endotoxin) on macrophages.

2.3.1. The role of B-lymphocytes in activation by endotoxin. In the present work stimulation of phosphatidylinositol turnover as well as induction of increased anti-bacterial and lysosomal activity by endotoxin did not require the presence of added lymphocytes. The addition of lymphocytes also did not influence the level of the response. It can be argued that the small proportion of B-lymphocytes possibly present among the 5% of the adherent cells that were not phagocytic played a role in mediating the effect of the endotoxin. It has been reported that B-cells are required for the stimulation by endotoxin of glucosamine uptake by guinea pig peritoneal macrophages (Wilton et al., 1975). The B-cells were either added to macrophage monolayers at a final number of 1 B-cell per 3 macrophages or unfractionated peritoneal exudate cells containing about 5% B-cells were used. The response in glucosamine uptake was measured after 72 hours of culture when the mitogenic effect of endotoxin on the B-lymphocytes will have been evident. The number of B-cells present was thus probably relatively large. In the present system the macrophage-monolayers were at least as well washed as in the experiments of Wilton et al. (1975) and thus not likely to contain more B-cells than their macrophage-monolayers which did not respond to endotoxin. The response in phosphatidylinositol

turnover occurred well before the endotoxin could have effected any B-cell proliferation. If B-cells were present they would not have been expected to take up the labelled myo-inositol as B-cells have been reported not to respond to mitogens including endotoxin by an increase in phosphatidylinositol turnover (Betel et al., 1974).

There is good evidence from other workers that B-cells are not essential for the activation of mouse macrophages by endotoxin. Alexander & Evans (1971) could render washed macrophage-monolayers, prepared from mouse peritoneal cells, cytotoxic by incubation with endotoxin. Recent experiments by Weinberg et al. (1978) and Rosenstreich et al. (1978a) have now confirmed quite convincingly that endotoxin acts directly on mouse macrophages. Thus it was shown that macrophages cultured in pure colonies from peptone-induced mouse peritoneal exudate cells in L-cell-conditioned medium became cytotoxic following the addition of endotoxin. The degree of the response was not influenced by the presence of B-lymphocytes (Weinberg et al., 1978). Rosenstreich et al. (1978a) showed that macrophages from a mouse strain with a genetically determined defect in B-cell responsiveness to endotoxin could still respond normally to endotoxin by enhanced production of lymphocyte activating factor and prostaglandins.

The conclusion from the evidence discussed above is that it is unlikely that B-cells mediated the effect of endotoxin in

the present experiments or contributed to the measured uptake of labelled myo-inositol.

2.3.2. Differences between mouse strains in macrophage responsiveness to endotoxin. Mouse strains vary in their responsiveness to bacterial endotoxins. Macrophages from the C3H/HeJ strain of mice are known to be highly resistant to the toxic effects of endotoxin (Glode et al., 1977) and require much higher doses of endotoxin compared with macrophages of other strains of mice, including the closely related strain C3H/HeN, to become cytotoxic (Weinberg et al., 1978) and start producing lymphocyte activating factor and prostaglandins (Rosenstreich et al., 1978b). No abnormality has been reported for the strain used here, C3H/Buf. It may be noted, however, that the concentration of endotoxin used in this study was relatively high compared with that used by some other workers for macrophage activation in vitro. Concentrations of around 10 µg/ml appear to be commonly used (Wilton et al., 1975; Weinberg et al., 1978; Rosenstreich et al., 1978b; Mørland & Kaplan, 1977) although higher concentrations are also recorded for example by Alexander & Evans (1971) who used 50 µg/ml. Endotoxins from different bacteria may vary in their potency and the endotoxin used in this study, E. coli 055:B5, has been shown to be less toxic to macrophages than endotoxins from some other strains of E. coli at the same concentration (Glode

et al., 1977). The concentration used in the present work (60 $\mu\text{g}/\text{ml}$ for 10^6 macrophages/ ml) was shown to be non-toxic and the final amount present was adjusted if necessary to the cell count present.

2.3.3. The effect of serum on macrophage activation by endotoxin. Serum-free medium was used in this study in order to eliminate the possible effects of serum components such as complement. Mørland & Kaplan (1977) have reported that endotoxin did not stimulate lysosomal enzyme activity or C3-receptor-mediated phagocytosis by mouse macrophages in vitro unless foetal calf serum (heat-inactivated) was present in the medium. As the serum was heat-inactivated the effect appears to have been mediated by serum factors other than complement. Many other workers have used serum-containing medium without testing for the effects of the serum but in the recent experiments of Weinberg et al. (1978) studying induction of macrophages cytotoxicity by endotoxin in vitro the activation as well as the cytotoxicity assays were performed in serum-free medium.

2.3.4. Effects of endotoxin on lysosomal enzyme activity of macrophages. Endotoxin has not been as much studied as some other agents with respect to an effect on lysosomal enzyme activity of macrophages. Pantalone & Page (1977) have stated that endotoxin does not induce mouse macrophages to

produce and release lysosomal acid hydrolases. However, in the paper that these authors refer to (Page et al., 1974) it was shown that exposure of mouse macrophages to endotoxin in vitro resulted in a marked increase in synthesis and secretion of acid phosphatase and some increase in production of N-acetylglucosaminidase whilst the levels of β -glucuronidase were unaffected. Previously Allison et al. (1973) had also reported an increase in synthesis and release of acid phosphatase and N-acetylglucosaminidase but no change in levels of β -glucuronidase or β -galactosidase following stimulation of mouse macrophages with endotoxin in vitro. In these experiments the enzyme levels were only measured after 72 hours of incubation and may therefore not be directly comparable to the present data. It may be noted, however, that the present experiments showed a more marked effect on the activity of acid phosphatase as compared with the effect on β -galactosidase or β -glucuronidase. The results of Mørland & Kaplan (1977) using endotoxin and mouse macrophages are somewhat similar. They found a very marked increase in intracellular levels of acid phosphatase already after 24 hours of in vitro culture with endotoxin; the increase in activity of β -glucuronidase was not very marked until after 48-72 hours of culture; cathepsin D levels were increased only after 72 hours but N-acetylglucosaminidase activity was unaffected. Only intracellular levels were measured.

The pattern of stimulation of lysosomal enzymes is thus clearly somewhat variable according to the experimental systems but acid phosphatase activity is consistently stimulated by endotoxin.

2.4 Lymphocyte-dependent activating effect of *C. parvum* on macrophages.

2.4.1. Macrophage activation by *C. parvum* requires lymphocytes or previous stimulation. The finding that stimulation by *C. parvum* of phosphatidylinositol turnover and lysosomal enzyme activity by macrophages required the presence of lymphocytes was not unexpected in view of previous reports (Christie & Bomford, 1975; Ghaffar & Cullen, 1976; Šljivić & Watson, 1977). Lymphocyte-independent stimulation of phosphatidylinositol turnover was observed only in a few experiments when an infection in the mouse colony had obviously had a stimulating and even activating effect on the macrophages. These macrophages had morphological characteristics of activated macrophages with increased granulation and marked and rapid spreading. They were also noted to be cytotoxic against tumour cells in vitro without further stimulation (Gwen Barrow, personal communication). As mentioned earlier Christie & Bomford (1975) also found that lymphocyte-independent activation of macrophages by *C. parvum* in vitro occurred only if the macrophages had been previously stimulated in vivo.

Schorlemmer et al. (1977b) have described a rapidly occurring release of lysosomal enzymes by macrophages with a corresponding decrease in intracellular levels after exposure to *C. parvum* in vitro in the absence of added lymphocytes. The washing procedure used to remove non-adherent cells appears

to have been less vigorous than that used in the present study but more significantly the dose of C. parvum was of a magnitude that was found to be toxic in the present work. It must also be emphasized that only release of preformed enzyme took place without evidence of stimulation of synthetic processes. The results presented here demonstrated a lymphocyte-dependent release of lysosomal enzymes in response to a non-toxic dose of C. parvum. This did not become evident until after 24 hours of culture and was not accompanied by a decline in intracellular levels. It is, therefore, implied that enhanced synthesis took place and the time course is comparable to that described for macrophage activation (see Table 22).

The relative number of lymphocytes used in the present study was much lower than that used in the experiments of Christie & Bomford (1975). The equal effectiveness of immune and non-immune lymphocytes also contrasts with the findings of Christie & Bomford. These authors found that induction of macrophage cytotoxicity by C. parvum in vitro could be achieved only in the presence of C. parvum-sensitized spleen cells at a ratio of 5 lymphocytes per 1 macrophage. A much lower number of lymphocytes was shown to be effective in some earlier experiments on antigen-dependent activation of macrophages. Thus Krahenbuhl & Remington (1971) found that the optimal number of Toxoplasma-immune spleen cells added with Toxoplasma-antigen to activate 2×10^6 guinea pig macrophages was 5 to 10×10^5 or 25-50% of

the number of macrophages. The activated macrophages inhibited the growth of Listeria. Simon & Sheagren (1972) also working with guinea pig macrophages and measuring anti-Listerial activity achieved activation with bovine gammaglobulin and bovine gammaglobulin-immune lymph node or peritoneal lymphocytes at ratios as low as 5-10% of the number of macrophages. These ratios of 5-10% or 25-50% are comparable to the ratio used here of 1 lymphocyte per 5 macrophages.

2.4.2. Identity of cell type involved in mediation of macrophage activation by C. parvum. Non-sensitized lymphocytes would, however, not have been expected to be effective mediators of activation from these previous reports. It was shown here that T-cell preparations from both C. parvum-immune and non-immune animals responded to exposure to C. parvum in vitro by an increase in DNA-synthesis. The non-immunized mice showed no serological evidence of an immune response to C. parvum. The reaction of the normal lymphocytes might, nevertheless, represent an immunologically specific response based on previous exposure to a cross-reacting antigen if the earlier response was either mainly limited to T-cells or too weak to be detected in a passive agglutination test. Alternatively C. parvum may have had a mitogenic effect and this would perhaps be in better agreement with the time course observed, with a maximum proliferative response of the T-cells

after 3 days of incubation with C. parvum. Antigen-induced proliferation of lymphocytes takes somewhat longer to become evident (Waithe & Hirschhorn, 1973). C. parvum has been reported to be mitogenic for B-cells but not for T-cells (Zola, 1975). It is thus conceivable that residual B-cells in the splenic T-cell preparations used here were responsible for the observed reaction. The response was, however, strictly dependent on the presence of the small proportion of macrophages added to the cultures. This suggests that the responding cells were T-cells as stimulation of B-cells with mitogens is not dependent on macrophages and may rather be depressed by the presence of macrophages in numbers that would stimulate T-cell responses (Rosenstreich & Oppenheim, 1976). In addition, endotoxin, which is a B-cell mitogen, did not stimulate the uptake of tritiated thymidine by the lymphocyte preparation in one experiment (this was not investigated further). C. parvum has recently also been reported to stimulate natural killer cells under certain circumstances in vivo although with different experimental conditions inhibition of natural killer cell activity could result (Ójo et al., 1978). It is not known whether a link exists between the stimulation of natural killer cells and activation of macrophages.

From the points mentioned above it can be seen that the identity of the cell type involved in the mediation of the stimulatory effects of C. parvum on the macrophages by the

splenic T-cell preparation is not quite certain. It appears, however, most likely to have been a T-cell.

2.4.3. Requirement for close contact between macrophages and lymphocytes. It was found that supernatants from unfractionated spleen cells cultured with or without C. parvum under conditions identical to those used in the phosphatidylinositol turnover assay could neither stimulate the uptake of labelled myo-inositol by macrophages nor replace T-lymphocytes in the stimulation of macrophages by C. parvum. Whole spleen cells were shown to have the same stimulatory effect as the purified T-cell preparation when added to macrophages with C. parvum. The failure of the supernatants to stimulate was, therefore, not caused by some kind of inhibition operating in an unfractionated cell population. The unfractionated spleen cells can be assumed to contain sufficient macrophages to support T-cell responses. These results indicate that either cell contact is required for the T-cell-mediated activation of macrophages by C. parvum or short-lived soluble factor(s) acting at a close range are involved. This is in contrast with the well-known ability of soluble lymphokines to activate macrophages in vitro (see "Introduction", 12.2.5. and Table 22). The culture period used in the present experiments (4 hours) was, however, probably too short to induce substantial release of lymphokines (Kasakura

& Lowenstein, 1965; Bartfeld & Atoyntan, 1971; Landolfo et al., 1977). The supernatants from these 4 hour cultures were not tested for lymphokine activity by other criteria. As the study of supernatants from longer term spleen cell cultures was met with difficulties, as described before, their properties were not tested further. It had, however, been noted in preliminary experiments that supernatants from mixed lymphocyte cultures of splenic lymphocytes from C3H/Buf and CFE mice showed MIF-activity for C3H/Buf macrophages whilst control supernatants from unmixed C3H/Buf or CFE lymphocytes were inactive. This did not correlate with the effect of these supernatants on the turnover of phosphatidylinositol by macrophages.

There are some recent reports suggesting a requirement for cell contact between T-cells and macrophages under certain experimental conditions. Thus Ando et al. (1976) found that PHA-stimulated rabbit lymphocytes stimulated the activity of β -galactosidase in rabbit peritoneal macrophages in vitro but the supernatants of these lymphocytes did not have this effect although there was evidence of MIF activity. Farr et al. (1977) showed that T-lymphocytes from mice infected with L. monocytogenes could induce the release of lymphocyte activating factor by macrophages in vitro. Cell to cell contact was required as well as homology at the I-A sub-region. Riisgaard

et al. (1978) have described stimulation of glucose oxidation in mouse macrophages by two, apparently separate, pathways involving either lymphocytes or their products. They showed that direct stimulation by immune lymphocytes and antigen required histocompatibility but was not affected by the presence of α -L-fucose. In contrast lymphocyte supernatants could stimulate macrophages from heterologous strains of mice but were ineffective in the presence of α -L-fucose which is known to inhibit the action of MIF.

2.4.4. Concluding remarks. As noted before the T-lymphocytes were not seen to bind C. parvum and did not respond to C. parvum by an increase in DNA-synthesis in the absence of macrophages. The T-cells were seen to collect around macrophages. The sequence of events leading to the stimulation of macrophage activity in the present experiments is, therefore, likely to have been as follows: initial uptake of C. parvum by macrophages resulted in presentation of C. parvum to lymphocytes accumulating around antigen- (or mitogen-) bearing macrophages. This led to stimulation of the lymphocytes with consequent activation of the macrophages by the lymphocytes.

3. THE ROLE OF NON-SPECIFIC CARBOHYDRATE-MEDIATED RECOGNITION IN MACROPHAGE ACTIVATION.

3.1.1. Binding of lymphocyte-independent activating agents. The question arises whether non-specific recognition (or perhaps "broad spectrum" recognition might be a more appropriate term) by macrophages can lead to their activation. When macrophage activation follows interaction with lymphocytes, lymphokines, immunoglobulins or complement this is most likely to be mediated by binding to specific receptor molecules. It is, however, difficult to see that specific receptor molecules should exist for all the various directly acting activating agents such as endotoxin, double-stranded RNA, pyran copolymer or Streptococcal cell walls (see "Introduction", 12.2.4.). It is possible that some of these agents cross-react with the receptors for the physiological mediators although it is not easy to see what some of these substances, composed of carbohydrates, lipids or nucleic acids, have in common with lymphokines, immunoglobulins or complement which are glycoproteins. Binding of bacterial lipopolysaccharide could easily be mediated by the non-specific carbohydrate-mediated recognition mechanism described here by virtue of its polysaccharide chain. Similarly Streptococcal cell walls are likely to be bound by the same mechanism and it may be speculated that the ribose of ribonucleic acid is involved in the binding of this substance.

The binding alone would, however, obviously not be sufficient to trigger activation as most of the bacteria bound in this way have no activating effect. It is known that the lipid A fraction of lipopolysaccharide has the full activating potential of the larger molecule (Alexander & Evans, 1971; Wahl et al., 1974). As lipid A is a glycolipid (Lüderitz et al., 1973) it might still attach to carbohydrate binding sites, the lipid component, however, appears to be essential for its action.

3.1.2. Triggering of macrophage activation in two stages. The possibility, therefore, arises that endotoxin acts in a manner not unlike that described for the action of cholera toxin on gut mucosal cells. The cholera toxin binds to the cell surface by one subunit of its molecule and the part of the molecule mediating the toxic effect is thought to be subsequently inserted into the lipid bilayer of the plasma membrane (Bennett et al., 1975; Cuatrecasas et al., 1976). The studies of Cuatrecasas and co-workers have also suggested the potential importance of movement of plasma membrane molecules in receptor-mediated stimulation of cells enabling reversible linkage of a surface receptor and membrane enzymes involved in the transmission of signals across the membrane (Cuatrecasas, 1974; Bennett et al., 1975; see also "Introduction", 8.1.4., 13.1.4.).

These observations might be of help in understanding the mechanisms involved in some recently reported studies on macrophage activation. Gordon et al. (1974b) had shown that phagocytosis of latex, which has no stimulatory effect on normal macrophages, was a potent stimulus for secretion of plasminogen activator if the macrophages had been previously stimulated with endotoxin in vivo. Very low amounts of endotoxin (0.5-1.0 ng/ml) have now been found to trigger the activation in vitro of macrophages previously exposed to sub-optimal doses of lymphokines in vitro (Ruco & Meltzer, 1978) or derived from mice inoculated with BCG (Weinberg et al., 1978). Pre-treatment with these small doses of endotoxin did not enhance subsequent responsiveness to lymphokines. The enhancing effect of the lymphokines lasted only for 24 hours (Ruco & Meltzer, 1978). It is conceivable that the first stimulus serves somehow to rearrange plasma membrane molecules in such a way that triggering by contact with a second stimulus is facilitated.

3.1.3. Defects in responses to macrophage activating agents. Recent studies have also shown that macrophages from mice that show genetically determined low responsiveness to the toxic and activating effects of endotoxin have some other defects as well. Thus they fail to respond to lymphokines in vitro by inhibited migration or development of cytotoxic activity;

cytotoxicity was also not induced by in vivo injection of BCG (Ruco et al., 1978; Tagliabue et al., 1978). It is implied that mechanisms involved in activation by endotoxin are closely linked to those mediating activation by other agents including lymphokines. Macrophages from these animals do respond to treatment with BCG in vivo or lymphokines in vitro by an increase in phagocytic activity and appear thus to be able to bind the mediating agents and respond to them in a limited way (Ruco et al., 1978). The possibility is thus raised that the defect lies in the transmission of the activating signal and it would be very interesting to study the phosphatidylinositol turnover response in macrophages from these low responder animals.

3.1.4. Concluding remarks. Whilst it is clearly too early to come to a conclusion about the role of non-specific (or "broad spectrum") carbohydrate-mediated recognition in macrophage activation it seems plausible that such recognition may mediate the binding of some lymphocyte-independent activating agents. Further interactions, perhaps involving different parts of the stimulating molecule or an ability to induce movement of plasma membrane molecules appear then to be required for the triggering of events, such as phosphatidylinositol turnover, leading to activation.

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APPENDIX

Publications from this work.

The characteristics of binding of *Corynebacterium parvum* to glass-adherent mouse peritoneal exudate cells

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SUMMARY

Corynebacterium parvum, strain 10390, whole organisms were shown to bind to the surface of glass-adherent mouse peritoneal exudate cells *in vitro*. An HCl extract and a lipid extract of the organism were both capable of inhibiting this binding. The attachment of organisms was not affected by trypsin treatment of the cells, indicating that the plasma membrane receptor is not cell-bound antibody in nature. The binding was inhibited by various sugars, most of which are major components of the cell wall of *C. parvum*. Removal of divalent cations prevented binding. At room temperature some binding occurred in the presence of magnesium ions alone, whereas both calcium and magnesium ions were required at 4°C. The possibility is discussed that the attachment of *C. parvum* to the plasma membrane of macrophages may lead directly to their activation.

INTRODUCTION

Anaerobic coryneforms (e.g. *C. parvum*) have been known for some years to bring about widespread effects on the lymphoid tissues, including a marked splenomegaly (Prévot & Van Phi, 1964), as well as *in vivo* activation of the cells of the mononuclear phagocyte system as shown by increased uptake of colloidal carbon and soluble protein antigen (McBride, Jones & Weir, 1974). Macrophages from stimulated animals also appear to retain antigen on their plasma membranes in greater amounts than found in unstimulated macrophages (Wiener & Bandieri, 1975). Peritoneal macrophages from *C. parvum*-treated mice show an increased ability to phagocytose syngeneic red cells (McBride, Jones & Weir, 1974) and also have a cytotoxic effect on mouse embryo fibroblasts in culture (Jones, McBride & Weir, 1975). *C. parvum* inhibits tumour growth in mice when injected with a variety of syngeneic tumours and the effect appears likely to be due to non-specific activation of macrophages by the coryneform organisms (Woodruff, McBride & Dunbar, 1974; Scott, 1974).

The way in which these organisms stimulate macrophages is not understood and the present communication provides evidence for a direct interaction between *C. parvum* and plasma membrane receptors of macrophages having specificity for sugar determinants in the cell wall of *Corynebacterium parvum*.

MATERIALS AND METHODS

Animals. CBA mice (SPF) age 5-10 weeks were obtained from the University animal breeding station.

Organisms. *C. parvum* (strain 10390) and *Propionibacterium freudenreichii* (strain 10470) were obtained from the National Collection of Type Cultures, Colindale. The organisms were grown in beef digest broth plus 3% glucose, harvested in Log phase and formalin killed as described by Dawes, Tuach & McBride (1974).

Antisera. Mouse anti-*C. parvum* serum was prepared by injecting mice IP with *C. parvum* (10390) at a dose of 0.7 mg in 0.1 ml saline. The animals were bled on day 10.

Collection of peritoneal exudate cells (PE cells). 2.5 ml of Dulbecco's phosphate-buffered saline (PBS) containing 10 i.u./ml

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heparin were injected IP into CBA mice and after light massage withdrawn by means of a syringe. The cell suspension, kept on ice, was centrifuged at 4°C at 250 g for 7 min and after removal of the supernatant resuspended in Medium 199 (Wellcome Research Laboratories, Beckenham) to give a final cell count of $2-4 \times 10^6$ /ml.

Preparation of monolayers. Suspensions of PE cells were layered on to 7 × 20 mm glass coverslips in 1.2 × 7.4 cm glass tubes (WR tubes). Tubes were incubated at 37°C for 2 hrs and non-adherent cells removed by washing with a stream of Dulbecco's PBS.

Binding assay. Coverslips with adherent PE cells were layered with a suspension of the appropriate organism at a concentration of 0.7 mg/ml in Dulbecco's PBS+B and incubated for 45 min at room temperature or for 90–120 min at 4°C. Non-attached organisms were washed away with Dulbecco's PBS and the coverslips air-dried, heat-fixed and stained by the Gram method. Microscopic examination of the monolayers was performed and PE cells with two or more attached organisms were counted. A total of approximately 200 cells were counted on each slide and the results expressed as the percentage of cells binding organisms.

Trypsin treatment and antibody coating. Trypsin (Difco Laboratories, Detroit, Michigan) at a concentration of 0.05% in Dulbecco's PBS+B was layered on to PE cell monolayers and incubated at 37°C for 15 min. Preparations were washed, Medium 199 added to the monolayers and left for a further 30 min at 37°C for receptor regeneration to occur.

Mouse anti-*C. parvum* (10390) serum, titre 1/384 (agglutination of *C. parvum*-coated latex particles as described by Woodruff, McBride & Dunbar, 1974), was used at a dilution of 1/15 and placed on the PE cell monolayers for 30 min at 37°C. The monolayers were then washed with Dulbecco's PBS and used in the binding assay.

EDTA treatment. A stock suspension (7 mg/ml) of *C. parvum* was mixed with EDTA to a final concentration of 10 mM, centrifuged, supernatant discarded and the procedure repeated twice. The organisms were finally resuspended to a concentration of 0.7 mg/ml in Dulbecco's PBS containing known concentrations of calcium and magnesium ions.

PE cell monolayers were incubated with 10 mM EDTA in Dulbecco's PBS for 10 min at 37°C, washed and incubated with Dulbecco's PBS containing calcium and magnesium ions at known concentrations. After 10 min at 37°C the monolayers were drained and the bacterial suspension in the appropriate ionic solution added as described before.

Inhibition studies. PE cell monolayers were overlaid with solutions of various carbohydrates (hexoses, pentoses, sugar acids, amino sugars, N-acetyl-derivatives and sugar alcohols) and organic acid salts (pyruvate, acetate and succinate) in Dulbecco's PBS+B. The concentration used was 10 mM as preliminary experiments had shown that this was the minimum inhibitory concentration for glucose and galactose. The culture supernatant of *C. parvum* 10390 containing cell wall material, HCl extract (Dawes, Tuach & McBride, 1974) and lipid extract of the whole organism were used undiluted. The lipid extraction was performed as described by McBride, Dawes & Tuach (1976), and the dried extract was dissolved in isotonic saline with the addition of NaOH and the pH finally adjusted to 7.2. After 15 min exposure to these materials at 4°C the monolayers were gently washed and the binding assay performed as described above.

RESULTS

Binding of whole C. parvum and cell wall extracts to PE cell monolayers

Mouse PE cell monolayers were exposed to whole *C. parvum* organisms *in vitro* at 4°C and room temperature. For comparison *P. freudenreichii* was included in this experiment. The latter organism has previously been shown to lack most of the biological activities associated with anaerobic coryneforms (McBride *et al.*, 1975b). *C. parvum* binds approximately eleven-fold more effectively than *P. freudenreichii* and the results are shown in Table 1. Each cell binds a greater number of organisms at room temperature than at 4°C.

TABLE 1. Binding of whole organisms to PE cell monolayers at 4°C and room temperature. Each figure is the mean of three replicates.

PE cell monolayers incubated with:	Percentage of cells binding organisms	s.e.
<i>C. parvum</i> 10390		
at 4°C	28.7	1.9
at room temperature	57.1	2.1
<i>P. freudenreichii</i> 10470		
at 4°C	2.7	0.6
at room temperature	4.7	0.8

Attempts were made to show which cell wall component was involved in the binding of the organisms by using HCl and lipid extracts of *C. parvum* known to be associated with the various biological activities of the organism (McBride, Dawes & Tuach, 1976). Preincubation of the PE cell monolayers with each extract inhibited binding of *C. parvum* whole organisms and the results are shown in Table 2. Inhibition by the HCl extract required the presence of calcium and magnesium ions (see below). The HCl extract gave a titre of 1/128 in counterimmunoelectrophoresis (Dawes, Tuach & McBride, 1974) against rabbit anti-*C. parvum* (10390) serum; the lipid extract showed no antigenic activity in this test.

Role of cell-bound antibody. Many animal species contain antibodies in their serum that react with antigens of anaerobic coryneforms (Woodruff *et al.*, 1974; McBride *et al.*, 1975a). It was, therefore, necessary to investigate the possibility that binding of *C. parvum* to the plasma membrane of adherent

TABLE 2. Inhibition of binding of *C. parvum* organisms to PE cell monolayers by cell wall extracts of *C. parvum*. Each figure is the mean of four replicates.

PE cell monolayers preincubated with:	Percentage of cells binding organisms	s.e.
Dulbecco's+B	16.4	1.3
HCl extract of <i>C. parvum</i> 10390	13.6	1.2
HCl extract of <i>C. parvum</i> 10390 supplemented with 2 mM Ca ²⁺ & Mg ²⁺	6.0	0.8
Lipid extract of <i>C. parvum</i> 10390	5.3	0.8

TABLE 3. Effect of trypsin treatment and antibody coating on the binding of *C. parvum* organisms to PE cell monolayers. Each figure is the mean of four replicates.

PE cell monolayers preincubated with:	Percentage of cells binding organisms	s.e.
Dulbecco's+B	55.3	2.2
Trypsin 0.05%	55.4	2.1
Mouse-anti-10390	90.2	1.5
Mouse-anti-10390 followed by trypsin 0.05%	50.5	2.0

PE cells may be due to cell bound antibody. Latex agglutination tests with *C. parvum*-coated latex particles and pooled serum from mice of the strain used in the *C. parvum* binding assay gave a negative result (titre \leq 1/6).

Trypsin is known to split immunoglobulins (Stanworth & Turner, 1973) and trypsin treatment has also been shown to destroy a C4 determinant on the macrophage plasma membrane. This determinant is regenerated within a few minutes after removal of trypsin (Ilgen & Burkholder, 1974) and thus trypsin treatment, whilst destroying surface bound proteins, does not appear to affect the integrity of the plasma membrane.

Table 3 shows the effect of trypsin treatment on binding of *C. parvum* to mouse PE cell monolayers at room temperature. Mouse anti-*C. parvum* serum can be seen to increase the binding of the organisms and binding is brought down to background levels by subsequent trypsin treatment indicating that under the conditions of the test trypsin, whilst able to destroy cell-bound antibody, did not reduce the binding of *C. parvum* to the plasma membrane of PE cells.

Binding inhibition studies. The cell wall material of *C. parvum* consists in large part of a high mol.wt. acidic polysaccharide, the major sugars of which are galactose, glucose and N-acetylglucosamine.

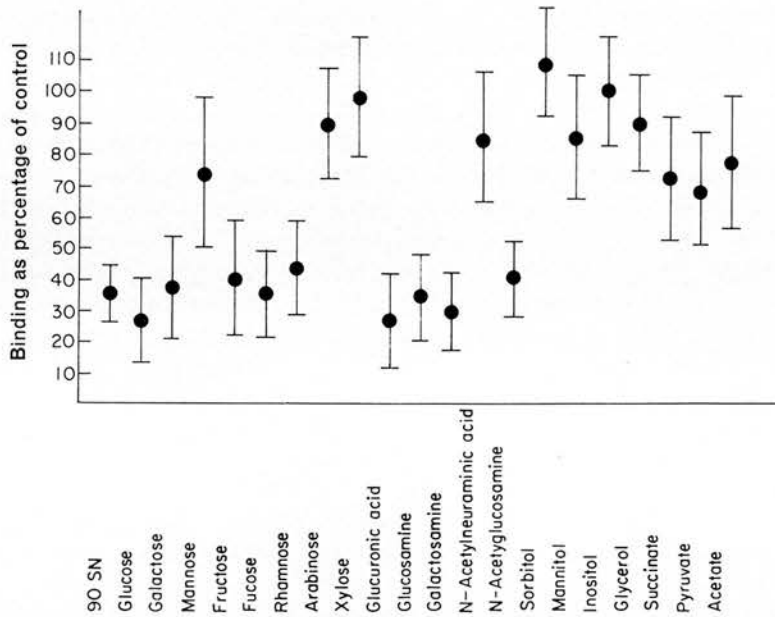


FIG. 1. Inhibition of binding of *C. parvum* organisms to PE cell monolayers by culture supernatant of *C. parvum* (90 SN), various carbohydrates and organic acids. Means of at least three replicates ± 2 s.e.

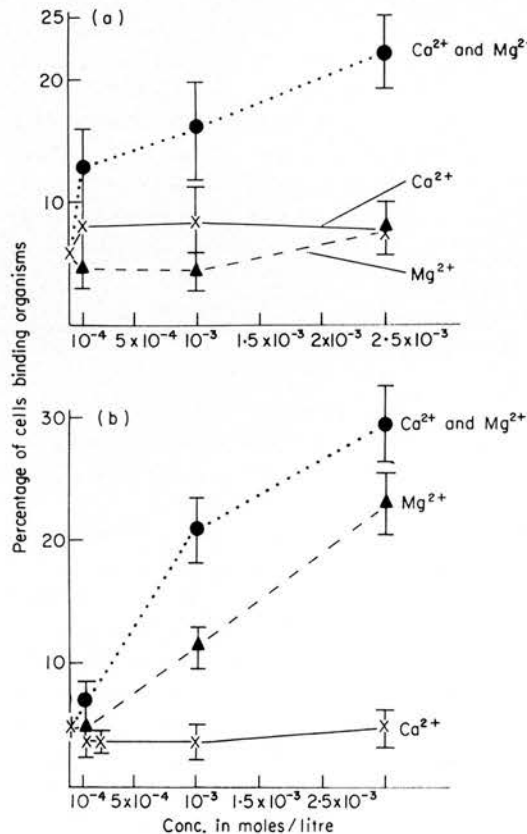


FIG. 2. The role of calcium and magnesium ions in the binding of *C. parvum* organisms to PE cell monolayers. (a) At 4°C: (b) at room temperature. Means of two to five replicates ± 2 s.e.

There are small amounts of various amino acids as well as a lipid component (Dawes, Tuach & McBride, 1974). The main antigenic constituent is N-acetylglucosamine (Dawes & McBride, 1975).

To investigate the possible role of these cell wall constituents in the binding of *C. parvum* to PE cells an inhibition assay was used as described in materials and methods. The culture supernatant of *C. parvum* (90 SN) and various carbohydrates and carbohydrate metabolites were tested and the results are shown in Fig. 1. The most effective inhibitor was the *C. parvum* cell wall material, followed by glucose, galactose, fructose, fucose, rhamnose, glucosamine, galactosamine, glucuronic acid and N-acetylglucosamine. None of the sugar alcohols or organic acid salts tried or N-acetylneuraminic acid had any inhibitory effect. All the inhibitory sugars with the exception of fructose and rhamnose are components of the cell wall of *C. parvum* and only one sugar known to be a cell wall component, i.e. mannose, was not a good inhibitor.

The role of divalent cations in binding of C. parvum to PE cells. Calcium and magnesium ions have been shown to be important in many biological processes acting for example as enzyme co-factors (Williams, 1970) and as promoters of phagocytosis (Stossel, 1973). The binding assay was therefore performed in the presence of known concentrations of calcium and/or magnesium ions after removing divalent cations from the PE cell plasma membrane and the bacterial cell wall by means of EDTA treatment.

Fig. 2 shows that binding of *C. parvum* to adherent PE cells does not occur in the absence of divalent cations, and that both calcium and magnesium are required for binding at 4°C, whereas binding at room temperature can be partly restored by magnesium alone.

DISCUSSION

This paper indicates that *C. parvum* strain 10390 binds to the plasma membrane of glass-adherent mouse PE cells having the morphological appearance of macrophages. In contrast the biologically inactive organism *P. freudenreichii* strain 10470 binds only to a few cells and then in very small numbers.

The binding can be inhibited by cell wall material derived from *C. parvum* culture supernatants. Biochemical analysis of this material has shown it to contain a variety of aldohexoses, amino sugars, N-acetyl derivatives and sugar acids (Dawes, Tuach & McBride, 1974). Various carbohydrates and carbohydrate metabolites were tested for their ability to inhibit binding of *C. parvum* whole organisms to adherent PE cells and the major cell wall components were found to be active inhibitors. Two other sugars, fructose and rhamnose, not previously detected in the cell wall material were inhibitory. Amongst the inhibitory substances N-acetylglucosamine has been shown to be an important antigenic determinant in the cell wall material as tested by radioimmunoassay (Dawes & McBride, 1975). A polysaccharide extracted by HCl hydrolysis (Dawes, Tuach & McBride, 1974) and a lipid extract (McBride, Dawes & Tuach, 1976) were both found to be inhibitory. The inhibition by the HCl extract shows the same dependence on divalent cations as the binding of the whole organism.

The possibility that the plasma membrane receptors on the PE cells were cell-bound antibody in nature was made unlikely by the failure of trypsin treatment (shown to destroy cell-bound antibody) to prevent binding of the organisms.

The role of divalent cations in bridge formation between carbohydrates has been recognized (Cook & Bugg, 1975). Removal of calcium and magnesium ions prevented binding of the organisms to PE cell monolayers and the binding at 4°C could be restored by addition of both cations, whereas at room temperature magnesium ions alone were capable of partly restoring the binding. This suggests the possibility that magnesium is acting as an enzyme co-factor (Osborn & Rothfield, 1971).

Recent work in this laboratory indicates that the interaction between *C. parvum* and PE cells *in vitro* leads to enhanced incorporation of ¹⁴[C]myoinositol into the membrane phospholipids (Ho, Ögmundsdóttir & Weir, unpublished results).

The interaction of neurotransmitters, hormones, drugs and bacterial toxins with plasma membrane receptors is generally recognized to be the signal that initiates the cell stimulation, drug or toxin effects. Internalization of the stimulant does not appear to be required in the case of Con A stimulation of lymphocytes (Betel & Van den Berg, 1972). The possibility exists that binding of *C. parvum* to the

plasma membrane of macrophages may be directly responsible for the wide range of activities shown by macrophages from *C. parvum* treated animals (Halpern, 1976).

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SPECIAL PAPER

Non-specific recognition mechanisms by mononuclear phagocytes

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Phagocytic cells in higher animals are capable of discriminating between foreign material and self components. They are also able to distinguish between self and alterations to self, as in effete or damaged tissue components or aberrant self constituents, such as tumour cells.

The recognition abilities that underlie this discriminating capacity must have evolved, at least in simple form, with the emergence of primitive species. Some form of self recognition mechanism is likely to have formed the basis of the abilities of colonial marine species (e.g. *Porifera*) to exclude unrelated forms from inclusion in their colonies. In addition, the cells lining the cavity of *Porifera* are capable of capturing micro-organisms present in the water drawn into the cavity. Phagocytosis also plays a role in the metamorphosis of insects in removing dead cells and disintegrated tissue. The role of invertebrate haemocytes in recognizing, walling off and engulfing foreign material is likely to be a development of these earlier, more primitive recognition mechanisms and begins to involve co-operation with soluble factors present in the haemolymph, acting as opsonins.

In vertebrates further specialization has occurred, involving co-operation between phagocytes and components of the immune system including antibodies, complement and lymphoid cells. The more primitive forms of phagocyte recognition appear, however, to have been retained and the cells appear to be able to recognize and bind foreign particulate material without involving the use of their more recently evolved receptors for the Fc component of the immunoglobulin molecule or the C3b component of the complement system.

There is a remarkable lack of understanding of the mechanisms that underlie these highly important recognition abilities of phagocytes. It is not known if binding of a bacterium to a phagocytic cell involves complex stereospecific receptors in the cell for components of the cell wall of the micro-organism or whether the binding depends on a simpler, relatively non-specific ability to recognize carbohydrate or other cell wall constituents of the bacterial cell wall. Other possible mechanisms include interactions depending on hydrophobic molecules, present in the foreign particle, inserting themselves in the phospholipid cell membrane of the phagocyte (Wilkinson, 1976). Hydrophilic polysaccharides of encapsulated bacteria thus resist such interactions as the surface of the phagocyte is hydrophobic in comparison.

Before considering the nature of non-specific types of binding to phagocyte plasma membranes, it would be useful to outline the general surface organization of membrane constituents (Nicolson & Poste, 1976).

Cell surface organization

Cell membranes contain lipids, proteins, oligosaccharides and polysaccharides in various combinations. Membrane phospholipids have hydrophilic ionic (or polar) heads that interact with water at the external and internal sides of the phospholipid bilayer. Their hydrophobic tails associate within the bilayer and exclude water. These molecules are capable of lateral movement in the membranes. Membrane proteins exist in hydrophilic and hydrophobic forms. The hydrophobic proteins (integral membrane proteins)

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are embedded to variable depths into the phospholipid bilayer and may span the membrane. In the latter situation they will have hydrophilic peptide sequences at either end of the molecule. Such molecules often have a carbohydrate chain at the external surface of the bilayer and are glycoproteins. Other non-structural proteins with hydrophilic properties are present at the external and internal surfaces of the lipid bilayer and are termed peripheral membrane proteins; they may, for example, be membrane-associated enzymes.

Peripheral membrane proteins can be removed from their ionic association with integral membrane proteins and glycolipids by high salt concentration and chelating agents. Their removal, in contrast to the integral proteins, does not affect the structural integrity of the cell membrane. The integral proteins can, like the phospholipids themselves, move laterally in the membrane. Membrane glycoprotein and glycolipids are arranged so that their carbohydrate residues are exposed at the external surface of the membrane. These molecules are likely to be of considerable importance in the present discussion as they are known to act as receptors for antibodies, hormones, lectins, viruses and other agents. Their lateral movement can result in the formation of patches or caps in which their distribution is altered from diffuse to a coalesced arrangement.

Microtubules together with microfilaments lie in the cytoplasm of the cell and control the mobility and distribution of cell surface receptors and are referred to as membrane-associated components.

The fact that different integral proteins and glycoproteins have considerably different rates of lateral movement suggests the importance of restricted mobility of some components so as to maintain an ordered display of patterns on the cell surface. Such cell-specific surface patterns could constitute an important mechanism for determining cell contact and recognition phenomena. The ability of some of these molecules to move rapidly in the cell membrane in response to environmental factors (e.g. lectin or antibodies, leading to capping) enables the reversible changes in cell surface organization to take place.

The redistribution of integral membrane proteins that accompanies phagocytosis in polymorphs and with the lectin concanavalin A in various cell types is believed to require the disruption (disassembly) of the microtubules that normally anchor the receptor in the cell membrane. Cyclic GMP appears to be required for assembly of microtubules and thus for the immobilization of receptors. Stimuli that increase cyclic GMP levels will therefore inhibit mobility of receptors and prevent capping (Oliver, 1975).

Interactions between cell membranes of phagocytes and bacteria

The ability of phagocytes to adhere to various surfaces and to particulate material in the absence of antibody or complement is a well-known phenomenon (Stossel, 1975). This ability is regarded as non-specific and the use of the term 'receptor' for the molecules involved in this reaction may be unjustified.

The role of divalent cations (particularly magnesium and calcium) in the binding and ingestion of non-opsonized particles such as albumin-coated oil particles has been established (Stossel, 1973). In contrast, monovalent ions appear to have relatively little effect on ingestion. We have confirmed the need for Mg^{2+} and Ca^{2+} ions in the binding of the immunological adjuvant *Corynebacterium parvum* to mouse peritoneal macrophages (Ögmundsdóttir & Weir, 1976). Removal of these ions by EDTA treatment prevented the binding of the organism at 4°C and this could be restored by the addition of both cations. At 20°C magnesium ions alone were capable of partly restoring the ability of *C. parvum* to bind to the PE cells. This suggests a complex series of interactions partly involving a carbohydrate- Ca^{2+} -carbohydrate bridge formation, and partly the possibility that magnesium is acting as an enzyme cofactor (see below).

It is of interest to note that the interaction of lymphocytes with macrophage receptors also requires the presence of divalent cations (Rosenthal *et al.*, 1975).

The role of carbohydrate constituents of the *C. parvum* cell wall in binding to the macrophage plasma membrane was investigated by a binding inhibition assay (Ögmundsdóttir & Weir, 1976). The cell wall material consists of galactose, glucose, mannose (trace amounts), fucose, N-acetyl galactosamine, N-acetyl glucosamine, sialic acids and uronic acids (Dawes, Tuach & McBride, 1974). All these sugars, with the exception of mannose and sialic acids, appear to be involved in the interaction. Fucose and rhamnose, which were not detected in the *C. parvum* cell wall, were also inhibitory, but none of the sugar alcohols or organic acids tried had any inhibitory effect (Fig. 1). Work in progress indicates that a

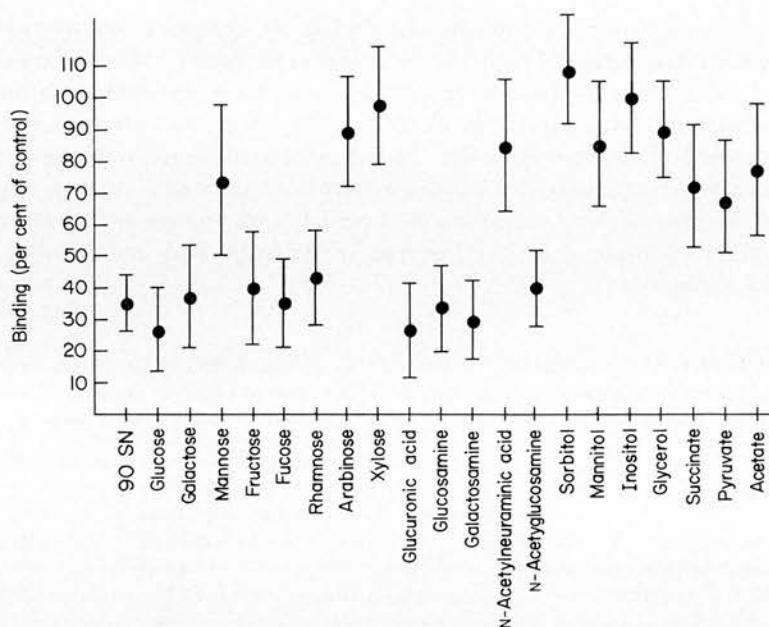


FIG. 1. Inhibition of binding of *C. parvum* organisms to mouse PE cell monolayers by culture supernatant of *C. parvum* strain 10390 (90 SN), various carbohydrates at 10 mM concentration in Dulbecco's PBS+B, and organic acids. Means of at least three replicates \pm two standard errors.

variety of Gram-positive and Gram-negative bacteria bind to macrophages in a similar way as *C. parvum*. Studies with a mutant strain of *Klebsiella aerogenes* deficient in galactose indicate that this sugar is involved in binding to the macrophage plasma membrane as the sugar fails to inhibit attachment of the organism to the macrophages whilst it is strongly inhibitory for a strain containing galactose. Similar results have been obtained with sugar-deficient mutants of *Salmonella typhimurium*, where the ability of a sugar to inhibit binding is directly related to its presence in the cell wall lipopolysaccharide (Freimer *et al.*, submitted for publication).

Insufficient data are available at present to identify the particular molecular structures within the macrophage plasma membrane that are involved in the 'non-specific' binding of bacteria and other particulate materials. It seems probable that integral membrane glycoproteins are the structures involved. They are known to be expressed in the macrophage plasma membrane and to be the site (receptors) of binding of various plant lectins which are recognized to be specific for glycoproteins (Sharon & Lis, 1972). ^{125}I -labelled concanavalin A, for example, binds to monolayers of macrophages as well as polymorphs. The receptors involved appear to be internalized, to some extent, during phagocytosis of unrelated particles such as latex beads or albumin-coated oil droplets and reappear within a few hours in culture (Oliver & Berlin, 1976). Another feature associated with binding of plant lectins with phagocytes is the lateral movement of the receptors in the plane of the membrane, resulting in their coalescence (cap formation). The role of microtubules and microfilaments in the movement of such receptors has been noted above (Oliver & Berlin, 1976).

A number of studies have been made on the effect of various enzymes and chemical treatments on the binding of particulate materials to phagocyte membranes, but the results do not allow the precise identification of the structures involved.

Our own studies on *C. parvum* binding to mouse peritoneal macrophages are summarized in Table 1 and show that most of the enzymes used are able to reduce binding of the micro-organism and that the binding ability returns after a period in culture. This is in contrast to the effect of sodium periodate, which has a more permanent effect on the binding sites, although binding can be restored by the borohydride reducing agent (Table 2).

Recovery of the binding sites after trypsin or β -galactosidase treatment requires the presence of

Ca^{2+} and Mg^{2+} . The need for magnesium ions for binding of *C. parvum* to occur at room temperature suggests that these enzymes digest the particular binding sites involved in the interaction with *C. parvum* cell wall material. In contrast, the effect of the phospholipases are surmised to be due to secondary distortive effects following their action on the membrane. The effect of a higher concentration of neuraminidase in increasing binding is probably due to a reduction in the negative charge of the macrophage membrane, thus enabling *C. parvum* with its negatively charged cell wall to come in closer contact with the macrophage. The effect of low concentrations of periodate on binding and its reversal by borohydride suggests the involvement of hydroxyl groups on the sialic acid, and possibly sugars such as galactose, in the binding site.

TABLE 1. The effect of enzymes on the binding of *C. parvum* to PE cell monolayers, recovery during 1 hr in tissue culture medium and the effect of EDTA on recovery

PE cell monolayers preincubated at 37°C for 12 min with:	PE cells binding <i>C. parvum</i> , per cent of control		
	Direct assay	Recovery incubation, 1 hr at 37°C, with culture medium containing:	
		No EDTA	10 mM EDTA
Trypsin (1 mg/ml)*	47.7±3.0	96.8±6.4	43.8±4.6
Pronase (250 µg/ml)*	47.7±3.3	35.7±3.3	43.9±3.7
β-galactosidase (0.05 eu/ml)†	53.9±3.1	87.2±6.4	62.1±5.5
Neuraminidase (5 u/ml)*	131.9±4.8	91.5±5.6	100.9±6.4
Phospholipase A (1.0 u/ml)‡	46.3±4.2§	91.1±7.4§	81.1±6.8§
Phospholipase C (20 µg/ml)*	42.6±2.6	91.3±5.0	81.3±5.0
Phospholipase D (0.05 eu/ml)¶	47.0±3.4	91.3±6.4	73.5±8.2
Dulbecco's PBS+B+glycerol (2.7%)	78.5±4.5	78.7±6.2	81.0±6.2
Dulbecco's PBS+B	100.0	91.5±4.0	95.5±4.0

Each figure is the mean of at least four replicates, data from two or more experiments pooled. Untreated controls showed 21.7% binding (s.d. = 3.6). Results are given as: (percentage binding after treatment/percentages binding of control) × 100, with the standard error shown as well.

* In Dulbecco's PBS+B, pH 7.2.

† In Dulbecco's PBS+B containing 0.05 mM Mn^{2+} , pH 7.2.

‡ Stored in 50% glycerol, diluted in physiological saline containing 10 mM Ca^{2+} , pH 7.4.

§ Per cent of control with corresponding concentration of glycerol.

¶ In Dulbecco's PBS+B, pH 5.6. Binding was shown not to be affected by the low pH alone.

No firm conclusion can be drawn from this data, but it seems possible that the binding sites on the macrophage plasma membrane are glycoprotein in nature. The binding may be between the hydroxyl groups of carbohydrate residues on membrane glycoproteins and carbohydrates in the bacterial cell wall, perhaps joined by Ca^{2+} bridges. The possibility exists that glycosyl transferase-carbohydrate interactions may also occur with magnesium ions acting as an enzyme cofactor.

Complex carbohydrates seem likely candidates as 'information-rich' molecules for a role in the intercellular adhesion, although additional evidence in support of this is desirable. It is known that the plasma membrane of liver cells has glycoprotein receptors specific for galactose residues on serum glycoproteins and is involved in the physiological regulation of their metabolism (Lunney & Ashwell, 1976). Hepatic cells are also able to recognize and bind lymphocytes which are deficient in their normal cell surface sialic acid residues after neuraminidase treatment (Woodruff & Gesner, 1969), suggesting that exposed sugar residues are involved in the binding.

The view has been expressed (Roseman, 1970) that glycosyl transferases and their acceptor molecules (i.e. substrates) are responsible for intercellular adhesion. These enzymes catalyse the transfer of sugars

TABLE 2. The effect of periodate on the binding of *C. parvum* to PE cell monolayers and reversal by borohydride

PE cell monolayers preincubated with:	PE cell binding <i>C. parvum</i> , per cent of control
Dulbecco's PBS+B, 5 min at 37°C	100.0
IO ₄ ⁻ , 1 mM, 5 min at 37°C	39.6±3.5
IO ₄ ⁻ , 1 mM, 5 min at 37°C, followed by Eagle's MEM, 60 min at 37°C	45.5±4.5
IO ₄ ⁻ , 1 mM, 5 min at 37°C, followed by BH ⁻ , 1 mM, 5 min at 37°C	81.7±5.4
IO ₄ ⁻ , 1 mM, 5 min at 37°C, followed by Dulbecco's PBS, 5 min at 37°C	44.6±4.5

Each figure is the mean of five to seven replicates, pooled data from three experiments. The standard error is shown as well.

from a sugar nucleotide to an oligosaccharide acceptor, and Roth, McGuire & Roseman (1971) have provided evidence that terminal β -galactopyranosyl groups are required to interact with specific surface glycosyltransferase for the adhesion of embryonic chicken neural retina cells. Glycosyltransferases, with the exception of sialyltransferases, require divalent cations for their activity; regulation of their activity may take place by changes in the local concentration of such ions at the cell surface.

The possible role of sugar molecules in interactions between cell surfaces by means of van der Waals forces has been emphasized by Nir & Anderson (1977), who calculated that the magnitude of the van der Waals forces between cell surfaces varied with their composition and that sugars made the biggest contribution in comparison with phospholipids, cholesterol and proteins.

Biological significance of macrophage recognition

The presence in the macrophage plasma membrane of glycoproteins and their ability to form bonds with carbohydrate cell wall material of micro-organisms indicates that the phenomenon may extend to other forms of macrophage interaction.

Proliferating cells such as fibroblasts and transformed cells (including cells transformed by oncogenic viruses or chemically induced tumour cells) are known to express carbohydrate groups capable of interacting with, and being agglutinated by, plant lectins (Sharon & Lis, 1972). Viral glycoproteins may also be inserted into the cell membrane of transformed cells and changes in surface sugar chain length and sequence may be induced (Hakomori & Murakami, 1968). Transformed cells show increased surface protease and glycosidase levels that may be responsible for cell surface alterations to glycoproteins. Polyoma virus-transformed hamster fibroblasts in culture are known to show an increase in surface galactosyltransferase, one consequence of which might be the transfer of galactose to oligosaccharide side-chains of membrane glycoproteins or glycolipids (LaMont, Gammon & Isselbacher, 1977). These findings suggest that one form of macrophage-target cell interaction may involve binding between macrophage plasma membrane sites and such altered carbohydrate groups. Such interactions could conceivably be associated with the control of cell proliferation, tissue regeneration and tumour cell growth.

The sites on transformed cells that react with different lectins are usually thought of as chemically and topographically distinct, although the glycoprotein receptor on human red cells for the kidney bean lectin has been extracted and found to inhibit the agglutination of red cells by lentil lectin. The explanation for such apparent lack of specificity may simply be that two lectins bind with different portions of the same oligosaccharide in the human erythrocyte surface. The likely topographic rearrangement resulting from increased mobility of binding sites for lectins in transformed cells, rather than the acquisition of new sites, has been mentioned above. Changes in the content or organization of membrane-

associated microfilaments and microtubules have been noted in transformed cells and such changes are likely to affect the mobility of membrane components. However, Hakomori & Murakami (1968) found that the glycolipids extracted from polyoma virus-transformed hamster kidney fibroblasts were able to inhibit cytoagglutination of the tumour cells by wheat germ lectin, whilst the glycolipid from normal cells was inactive. They suggested that this was due to structural changes, such as incomplete carbohydrate chains in the glycolipids of the malignant cell.

The possibility that interactions of this type occur between tumour cells and macrophages is suggested by work in progress, in which the binding of mouse macrophages to methylcholanthrene fibrosarcoma cells is inhibited by 10 mM glucose (Weir, Grahame & Ögmundsdóttir, unpublished results). Our observations, that mouse peritoneal exudate macrophages activated *in vivo* by *C. parvum* had a cytotoxic effect on proliferating syngeneic mouse embryo fibroblasts, could be explained by such a mechanism (Jones, McBride & Weir, 1975), as could the cytotoxic effects of activated macrophages on tumour cells (Ghaffar *et al.*, 1974). It is also well known that macrophages are closely associated with growing tumour cells, particularly at the periphery of such tumours. Replicating lymphocytes are known to be highly susceptible to the cytostatic effects of activated macrophages and the role of such cells in controlling DNA synthesis and cell replication is of considerable interest (Keller, 1975).

The possibility arises that the increase in size of the macrophage during activation, as seen by its increase in ruffled membrane and spreading on a glass surface, is associated with changes in the density distribution of sialic acids in the membrane. The charged sialic acid groups are believed to be responsible for a long-range electrostatic repulsion between cells, and an increase in cell size (and thus a charge density decrease) of cultured HeLa cells appears to be associated with reduction in these forces (Denman, Vakaet & Bruyneel, 1976). Thus the enlarged activated macrophage might be expected to show a similar reduction in long-range electrostatic repulsion, which would enable it to adhere more effectively to negatively charged particles like micro-organisms or other tissue cells.

Whether or not interactions of this type play any role in the induction of the immune response is a matter of speculation. The ability of macrophages to bind and phagocytose bacteria such as *C. parvum* in the way described suggests the possibility of such a role. Clearly many complex antigens contain carbohydrate groups as part of their molecular structure and could therefore be bound and internalized by the macrophage in the same way as *C. parvum*. Such a possibility would implicate the binding phenomenon we have described in the initiation of immunity.

Whether the interaction of lymphokines (such as MIF) with macrophages takes place by similar binding sites cannot be deduced from the available evidence. The receptor for MIF is known to be affected by a variety of enzymes, to recover after such treatment and in one report is said to be destroyed specifically by a fucosidase (Remold, 1973). The effect of neuraminidase is to increase the uptake and response of guinea-pig macrophages to MIF (Leu *et al.*, 1972).

In contrast to these findings, the receptor on the macrophage membrane for the IgG Fc component is stable to proteolytic enzymes and neuraminidase (Arend & Mannik, 1973), whilst that for C3b is destroyed by trypsin. Although it seems likely that these various receptors are rather different in a number of respects from that described for *Corynebacterium parvum*, the available evidence does not allow firm conclusions to be reached.

In our working hypothesis the binding of *C. parvum* to mouse macrophages, involving carbohydrates of the microbial cell wall and possible glycoprotein receptor sites on the macrophage plasma membrane, represents a primitive recognition mechanism enabling phagocytes to bind micro-organisms and cells prior to engulfment. We believe that this mechanism developed before the more specialized and more specific mechanisms involving cell-bound opsonins (including immunoglobulin), complement factors and sites for lymphocyte products that trigger macrophage activation, migration, inhibition and chemotaxis.

The phenomenon we have described may play a role in macrophage binding interactions with a variety of micro-organisms, carbohydrate antigenic determinants, transformed tissue cells and proliferating and neoplastic cells and is depicted schematically in Fig. 2. The possible physiological consequences of such interactions, such as effects on cell proliferation, tissue regeneration, tumour cells growth and induction of the immune response remain a matter for future investigation.

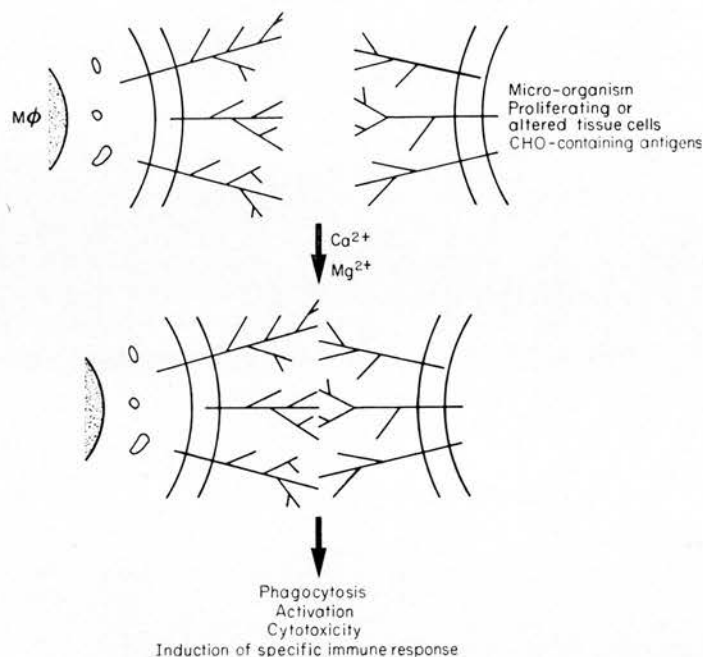


FIG. 2.

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BINDING OF MICROORGANISMS TO THE MACROPHAGE PLASMA MEMBRANE; EFFECTS OF ENZYMES AND PERIODATE

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Summary.—The nature of the binding of *C. parvum* organisms to the surface of glass-adherent mouse peritoneal exudate cells *in vitro* was studied using pretreatment of the cells with various enzymes and periodate. Trypsin, pronase, β -galactosidase, phospholipases A, C and D and periodate all caused a decrease in binding to 40–60% of untreated control. Neuraminidase led to a 30% increase in binding. The binding ability returned to normal after 1 h at 37° in culture medium following exposure to all the enzymes apart from pronase, which apparently could not be removed effectively by washing. The presence of EDTA in the medium inhibited recovery from treatment with trypsin and β -galactosidase; return to normal after exposure to phospholipases A, C and D was slightly affected, whereas recovery from treatment with neuraminidase was unaffected. Cells that had been exposed to periodate did not regain normal binding ability after 1 h in tissue culture medium but the effect could be reversed chemically by treatment with borohydride. The role of different plasma membrane components in non-specific cellular recognition is discussed.

In a previous communication (Ögmundsdóttir and Weir, 1976) we described the binding of *Corynebacterium parvum* to the plasma membrane of macrophage monolayers prepared from peritoneal exudate cells. The results indicated that the binding involved a recognition mechanism for carbohydrates on the bacterial cell wall as it was inhibited by various cell wall sugars, and studies in progress have shown that a variety of Gram-positive and -negative bacteria can attach to the macrophage membrane, probably by the same mechanism. The binding requires the presence of Ca^{2+} and Mg^{2+} ions, as has been found for the phagocytosis of unopsonized albumin particles (Stossel, 1973). It is not mediated by cell-bound antibody.

The nature of the binding to the macrophage plasma membrane was investigated further by subjecting the cells to treatment with a variety of enzymes and chemicals. Enzymes have been widely

employed in the study of the structure and function of cell membranes, including membrane receptors (Singer and Nicolson, 1972; Bretscher, 1973; Cuatrecasas, 1974; Steck, Fairbanks and Wallach, 1971; Zwaal *et al.*, 1975; Weiss *et al.*, 1972). Mild treatment with periodate removes the terminal two carbon atoms from sialic acid and oxidizes the alcohol group on carbon 7 to aldehyde. Subsequent treatment with borohydride reduces this aldehyde group to an alcohol group (Van Lenten and Ashwell, 1971). The rest of the sialic acid molecule and other carbohydrates are not affected under these conditions. These chemicals have been used in the study of membrane carbohydrates (Blumenfeld, Gallop and Liao, 1972).

MATERIALS AND METHODS

Animals.—C3H mice (SPF) age 8–12 weeks were obtained from the departmental breeding colony.

Organisms.—*C. parvum* (Strain 10390) was obtained from the National Collection of Type Cultures, Colindale. The organisms were grown anaerobically in horse digest broth +3% glucose, harvested in log phase and formalin-killed as described by Dawes, Tuach and McBride, (1974).

Enzymes.—The enzymes were obtained from the following sources: pronase (*Streptomyces griseus*), β -galactosidase (*Saccharomyces fragilis*) and neuraminidase (*V. cholerae*) were obtained from British Drug Houses, Poole, Dorset; phospholipase A (*Vipera russeli*), phospholipase C (*Cl. perfringens*) and phospholipase D (cabbage) from Koch Light Laboratories, Colnbrook, Buckinghamshire; and trypsin from Difco Laboratories, Detroit, Michigan. They were used without further purification.

Collection of peritoneal exudate cells (PE cells).—2.5 ml of Dulbecco's phosphate-buffered saline (Dulbecco's PBS) containing 10 i.u. heparin/ml were injected i.p. into C3H mice and after light massage withdrawn by means of a syringe. The cell suspension, kept on ice, was centrifuged at 4° at 250 g for 7 min and, after removal of the supernatant, resuspended in Medium 199 or Eagle's MEM (Wellcome Research Laboratories, Beckenham) without serum to give a final cell count of approx. $4-6 \times 10^6$ /ml.

Preparation of monolayers.—Suspensions of PE cells were layered on to 7 × 20 mm "flying" glass coverslips in 1.2 × 7.4 cm glass tubes (W.R. tubes), 0.125 ml per coverslip. Tubes were incubated at 37° for 1½ h and non-adherent cells removed by washing 7 times with Dulbecco's PBS containing Ca^{2+} and Mg^{2+} ions, (Dulbecco's PBS+B). Each coverslip then contained approx. 2.5×10^5 cells.

Treatment of PE cell monolayers with enzymes and chemicals.—PE cell monolayers were exposed to the appropriate enzyme solution for 12 min at 37°. The preparations were then washed 5 times with Dulbecco's PBS+B. The highest non-toxic concentration for each enzyme was determined in preliminary experiments and used for subsequent experiments. Sodium periodate (NaIO_4) and potassium borohydride (KBH_4) were used at a concentration of 1 mM in Dulbecco's PBS. The PE cell monolayers were incubated with either of these solutions for 5 min at 37°, and then washed with Dulbecco's PBS+B.

After the monolayers had been treated in the way described, they were either used directly in the binding assay or incubated for 1 h at 37° with tissue culture medium (199 or Eagle's MEM without serum) with or without 10 mM EDTA before adding the bacterial suspension.

Binding assay.—The PE cell monolayers were overlaid with a suspension of *C. parvum* organisms in Dulbecco's PBS+B at a concentration that gave approx. 4×10^9 bacteria per

coverslip. The coverslips were incubated at 4° for 2 h. They were then washed thoroughly, air-dried, heat-fixed and stained by the Gram method. Microscopic examination of the monolayers was performed and the percentage of cells with two or more organisms attached determined. Approx. 200 cells were counted on each slide. The final results are expressed as: (percentage binding after treatment/percentage binding of untreated controls) × 100.

RESULTS

Effect of enzyme treatment on the binding of C. parvum organisms to PE cell monolayers and recovery from the effect

The effect of preincubation of PE cell monolayers with various enzymes is shown in Table I. It can be seen that all the enzymes used, apart from neuraminidase, caused a decrease in binding to 40–60% of that of untreated controls. Neuraminidase led to a 30% increase in binding. Higher concentrations did not have a greater effect unless they were also toxic for the cells, as shown by decreased adherence to the coverslips and decrease in trypan blue exclusion. β -galactosidase and phospholipase D could not be completely dissolved at higher concentrations.

If the monolayers were incubated for 1 h at 37° in tissue culture medium, after washing away the enzyme solution but before adding the organisms, the binding ability had returned to control levels (Table I). Pronase-treated monolayers were an exception, however, as the effect of this enzyme increased during this incubation period.

Bearing in mind the importance of Ca^{2+} and Mg^{2+} ions for the binding of *C. parvum* to PE cells it was of interest to investigate the effect of the absence of free Ca^{2+} and Mg^{2+} ions on the recovery from enzyme treatment. The "recovery" incubation was therefore also performed with tissue culture medium containing 10 mM EDTA. This led to different results depending on which enzyme the cells had been exposed to. Thus it can be seen that recovery from treatment with trypsin and β -galactosidase was completely prevented in the

TABLE I.—*The Effect of Enzymes on Binding of C. parvum to PE Cell Monolayers, Recovery during 1 h in Tissue Culture Medium and the Effect of EDTA on Recovery*

PE cell monolayers preincubated at 37°C for 12 min with	PE cells binding <i>C. parvum</i> , percentage of control					
	Direct assay		Recovery incubation, 1 h at 37° with culture medium containing			
			No EDTA		10 mM EDTA	
		s.e.		s.e.		s.e.
Trypsin 1 mg/ml†	47.7	3.0	96.8	6.4	43.8	4.6
Pronase 250 µg/ml†	47.7	3.3	35.7	3.3	43.9	3.7
β-galactosidase 0.05 e.u./ml‡	53.9	3.1	87.2	6.4	62.1	5.5
Neuraminidase 5 u/ml‡	131.9	4.8	91.5	5.6	100.9	6.4
Phospholipase A 1.0 u/ml§	46.3*	4.2	91.1*	7.4	81.1*	6.8
Phospholipase C 20 µg/ml†	42.6	2.6	91.3	5.0	81.3	5.0
Phospholipase D 0.05 e.u./ml¶	47.0	3.4	91.3	6.4	73.5	8.2
Dulbecco's PBS+B+glycerol 2.7%	78.5	4.5	78.7	6.2	81.0	6.2
Dulbecco's PBS+B	100.0		91.5	4.0	95.5	4.0

Each figure is the mean of at least 4 replicates, data from two or more experiments pooled.

* Percentage of control with corresponding concentration of glycerol.

† In Dulbecco's PBS+B, pH 7.2.

‡ In Dulbecco's PBS+B containing 0.05mM Mn²⁺, pH 7.2.

§ Stored in 50% glycerol, diluted in physiological saline containing 10mM Ca²⁺, pH 7.4.

¶ In Dulbecco's PBS+B, pH 5.6. Binding was shown not to be affected by the low pH alone.

absence of free divalent cations, whereas return to normal following treatment with the other enzymes is only slightly affected (*viz.* phospholipases A, C and D) or unaffected by EDTA (*viz.* neuraminidase) (Table I). Pronase-treated cells showed a higher degree of binding after "recovery" in EDTA-containing medium than medium without EDTA and this was similar to the binding observed directly after exposure to pronase. These findings were confirmed using a higher and more toxic concentration of pronase (results not shown). It is known that some components of pronase require Ca²⁺ for their stability (Awrad *et al.*, 1972).

The effect of periodate on the binding of C. parvum to PE cell monolayers and reversal by borohydride

Pretreatment of the PE cell monolayers with periodate had the same effect as the enzymes (apart from neuraminidase), as is shown in Table II. The viability of the cells was not affected. Incubation with periodate for 15 min at room temperature had a similar effect, but exposure for longer than 5 min at 37° reduced the viability of the cells. Incubation for 1 h at 37° in tissue culture medium after the periodate had been washed away but before adding the organisms did not lead to a return to normal binding ability. On

TABLE II.—*The Effect of Periodate on the Binding of C. parvum to PE Cell Monolayers and Reversal by Borohydride*

PE cell monolayers preincubated with	PE cells binding <i>C. parvum</i> percentage of control	
		s.e.
Dulbecco's PBS+B, 5 min, 37°	100.0	
IO ₄ - 1mM, 5 min, 37°	39.6	3.5
IO ₄ - 1mM, 5 min, 37°, followed by Eagle's MEM, 60 min, 37°	45.5	4.5
IO ₄ - 1mM, 5 min, 37°, followed by BH ₄ ⁻ , 1mM, 5 min, 37°	81.7	5.4
IO ₄ - 1mM, 5 min, 37°, followed by Dulbecco's PBS, 5 min, 37°	44.6	4.5

Each figure is the mean of 5-7 replicates, pooled data from 3 experiments.

the other hand the effect of periodate could be reversed if periodate-treated cells were exposed to borohydride for 5 min at 37° before adding the bacterial suspension.

DISCUSSION

The results indicate that the binding of *C. parvum* organisms to mouse peritoneal macrophages is decreased by pretreatment of the cells with proteolytic and lipolytic enzymes and one glycolytic enzyme. This contrasts with the results of studies made on other, and probably more specific macrophage receptors. Thus the Fc receptor is destroyed by phospholipases A and C, but not D, and binding of immunoglobulin is increased following treatment with trypsin or neuraminidase (Davey and Asherson, 1967; Arend and Mannik, 1973). The complement receptor is destroyed by trypsin (Huber *et al.*, 1968; Lay and Nussenzweig, 1969). The response to macrophage migration inhibitory factor (MIF) is abolished by α -L-fucosidase and chymotrypsin treatment but increased following exposure to neuraminidase (Leu *et al.*, 1972; Remold, 1973). The inhibitory effect of macrophages on lymphoblastogenesis induced by lipopolysaccharide (LPS) or phytohaemagglutinin (PHA) was abolished by pronase treatment of the macrophages but not affected by neuraminidase or β -galactosidase (Baird and Kaplan, 1977). Pronase has also been found to inhibit the induction of macrophage cytotoxicity by a lymphocyte product, whereas trypsin and papain had no effect. The expression of cytotoxicity was not affected if the cells were treated with any of these three enzymes after macrophage activation (Dy *et al.*, 1976). However, the assays involved in the cytotoxic and cytostatic tests require long incubation periods and thus it seems possible that the membrane receptors concerned may have recovered during culture; this interpretation is supported by our present results. The fact that pronase alone seems to have an effect in these long-term assays may be explained by our

finding that this enzyme cannot readily be removed from the macrophage membrane by washing. Thus the effect of pronase increased during 1 h in culture medium.

After 1 h in tissue culture medium the cells had recovered from treatment with all the enzymes except pronase. Differences between macrophage monolayers treated with different enzymes were observed after a "recovery" period in the presence of EDTA. Thus recovery from treatment with trypsin and β -galactosidase was completely prevented in the absence of divalent cations, whereas return to normal following treatment with the other enzymes is only slightly affected (*viz.* phospholipases A, C and D) or unaffected by EDTA (*viz.* neuraminidase). This indicates that the mechanisms of repair and resynthesis of the phospholipids may not be as dependent on the presence of divalent cations as those involved in the repair of glycoproteins. Alternatively the membrane lipids may play a relatively less important role in the binding than the surface glycoproteins. Destruction of membrane lipids is known to have secondary effects on membrane proteins and many enzymes including glycosyltransferases are lipid-dependent (Singer and Nicolson, 1972; Labow, Williamson and Layne, 1973). Hydrolysis of the outer half of the lipid bilayer, as brought about by phospholipase action on intact cells, also leads to distortion of the membrane (Allan *et al.*, 1975).

The only enzyme that caused an increase in the binding of *C. parvum* to macrophages was neuraminidase. This is likely to be caused by a decrease in net negative charge on the cell surface, as the bacterial cell wall appears to be negatively charged (Dawes, Tuach and McBride, 1974).

It is unlikely that the enzymes exert their effect by binding to the cell membrane and thereby block the attachment of organisms in our system. The enzymes were removed by washing before the organisms were added and the successful removal of the enzymes, apart from

pronase, is indicated by recovery of the binding sites during 1 h in tissue culture medium. It is improbable that the recovery was due to internalization of the enzymes that had blocked binding, as this would require that EDTA interfered with the internalization of some enzymes but not others.

The decrease in binding caused by treatment with IO_4^- and reversal of this effect by subsequent exposure to BH_4^- suggests the involvement of hydroxyl groups on the plasma membrane in the binding of the organisms. The hydroxyl groups on the terminal three carbon atoms of sialic acids would seem to be relatively important, as only these are affected by periodate treatment under the mild conditions used. Other sugars such as galactose would also be expected to provide hydroxyl groups and this is suggested by the decrease in binding following treatment with β -galactosidase. Hydroxyl groups on galactose could also mediate the binding observed after exposure to neuraminidase, as sialic acids are frequently bound to this sugar in glycoproteins and glycolipids (Tuppy and Gottschalk, 1972). As the effect of mild treatment with periodate is so limited, it is not clear why this prevents the binding of *Esch. coli* to mucosal cells, which on other evidence seems to be mediated by mannose on the plasma membrane (Ofek, Mirelman and Sharon, 1977).

Periodate increases the response of macrophages to MIF, although this response has been found to be abolished by treatment with α -L-fucosidase (Remold, 1977; Remold, 1973). Lymphocytes undergo blastogenesis following treatment with periodate. This is inhibited by subsequent reduction by borohydride, after which the cells respond normally to phytohaemagglutinin (Zatz *et al.*, 1972). In contrast to enzyme-treated cells, macrophages that had been treated with IO_4^- did not regain their normal ability to bind *C. parvum* during 1 h in tissue culture medium. Periodate causes only a modification of sialic acids but does not remove the whole

molecule. Replacement by normal sialic acid might be expected to occur at an approximately normal rate of turnover, as glycoproteins containing sialic acid modified by treatment with periodate and borohydride show normal metabolic behaviour (Van Lenten and Ashwell, 1971). The turnover of proteins in the macrophage plasma membrane has been shown to have a half time of 7–8 h (Nachman, Ferris and Hirsch, 1971). Repair following removal of molecules by enzymatic action seems to be rapid. Trypsin treatment destroys a C4 determinant on the macrophage plasma membrane. This determinant is regenerated within a few minutes after removal of trypsin (Ilgen and Burkholder, 1974).

The results presented here and in our previous communication suggest that the binding of *C. parvum* to the macrophage cell membrane occurs by way of a non-specific recognition mechanism for carbohydrates. The binding requires an intact plasma membrane.

Whilst no firm conclusions can be drawn on the precise nature of the binding sites for *C. parvum* on the macrophage plasma membrane, the data indicate the probable importance of carbohydrate residues on glycoprotein molecules. These may interact with carbohydrate in the bacterial cell wall by means of Ca^{2+} bridges. The possibility that complex (information-rich) carbohydrates may be involved in intercellular adhesion has been pointed out by Roseman (1970) and Edwards (1977). Glycosyltransferases may play a part in stabilizing the interaction. Despite the potential importance of such mechanisms the evidence in support is slender at present. The possible role of sugar molecules in interaction between cell surfaces by means of van der Waals forces has been emphasized by Nir and Anderson (1977), who calculated that the magnitude of such forces varied with the composition of the cell surface, and that sugars made the largest contribution in comparison with phospholipids, cholesterol and proteins.

Whether or not binding interaction by

macrophages such as we have described extend to other forms of macrophage adherence remains to be elucidated. It is conceivable that interaction of this type may be responsible for macrophage recognition and attachment to a variety of carbohydrate-containing surfaces such as transformed or altered tissue cells, microorganisms and carbohydrate antigenic determinants.

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THE ROLE OF CELL WALL CARBOHYDRATES IN BINDING OF MICROORGANISMS TO MOUSE PERITONEAL EXUDATE MACROPHAGES

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Freimer, N. B., Ögmundsdóttir, H. M., Blackwell, C. C., Sutherland, I. W., Grahame, L. & Weir, D. M. The role of cell wall carbohydrates in binding of microorganisms to mouse peritoneal exudate macrophages. *Acta path. microbiol. scand. Sect. B*, 86: 53-57, 1978.

The recognition by macrophages of unopsonized bacteria was studied, employing a binding assay, performed at 4° C. Various Gram positive and Gram negative bacteria were shown to bind to glass-adherent mouse peritoneal exudate cells under these conditions, *Str. pneumoniae* being the only exception. The binding could be inhibited by pretreatment of the macrophage monolayers with various monosaccharides. The role of particular components of the bacterial cell wall in binding was examined further using different strains of *K. aerogenes* and *S. typhimurium* with a known cell wall composition and mutant strains deficient in certain sugars. The ability of a particular constituent to inhibit binding was found to correlate closely with its presence in the bacterial cell wall. It is concluded, that this form of binding, mediated by cell wall carbohydrates represents a primitive recognition mechanism enabling phagocytes to bind microorganisms.

Key words: Macrophages; recognition; bacterial cell wall carbohydrates; *Salmonellae*; microbial pathogenicity.

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The ability of phagocytes to adhere to various surfaces, to microorganisms and other particulate material in the absence of antibody or complement is a well known phenomenon (see Stossel 1975). The mechanisms underlying cell surface interactions of this type are poorly understood and in a previous report we showed the importance of carbohydrate constituents of the cell wall of the anaerobic coryneform *Corynebacterium parvum* in

binding to mouse peritoneal exudate macrophages (Ögmundsdóttir & Weir 1976). The plasma membrane receptors for the cell wall carbohydrates appear to be glycoprotein in nature and the binding is dependent on divalent cations (Ögmundsdóttir *et al.* 1977).

Macrophage recognition abilities are of considerable potential importance in the understanding of immune mechanisms in infective disease and macrophage target cell interaction with altered tissue cells. In this report we have studied the role of cell wall carbohydrates of a variety of Gram +ve and -ve microorganisms in binding to mouse macrophages using mainly organisms with defined cell wall composition.

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MATERIALS AND METHODS

Animals: C3H mice (SPF) age 5 to 10 weeks were from the departmental breeding colony.

Organisms: *Staphylococcus albus*, *Staphylococcus aureus*, *Streptococcus viridans*, *Streptococcus pyogenes*, *Escherichia coli*, and *Bacillus anthracoides* were obtained from the departmental teaching collection. *Pseudomonas aeruginosa* NCTC 578, both mucoid and non-mucoid strains, were kindly provided by Dr. John Govan. *Klebsiella aerogenes* serotype 2, NCTC 5055, a non-capsulated mutant M2, and a rough mutant M10B as well as *Salmonella typhimurium* SL 1542 with a complete lipopolysaccharide (LPS) and rough mutants having incomplete LPS: SL 1096 (*RfaK* chemotype Rb), SL 1099 (chemotype Rc) and SL 1102 (*rfaE*, chemotype Re) were from the strain collection of Dr. Ian Sutherland. The organisms were grown in horse digest broth or nutrient broth, harvested in log phase and killed by 24 h exposure to 0.5% formalin at 4° C. When living organisms were used they were stored at 4° C in 0.9% saline. Counting was performed in a Neubauer chamber 0.01 mm depth.

Collection of peritoneal exudate cells (PE cells): 2.5 ml of Dulbecco's phosphate buffered saline (PBS) containing 10 IU/ml heparin, were injected i.p. into C3H mice and after light massage withdrawn by means of a syringe. The cell suspension, kept on ice, was centrifuged at 4° C at $250 \times g$ for 7 minutes and, after removal of the supernatant, resuspended in Eagle's medium MEM (Wellcome Research Laboratories, Beckenham, England) without serum to give a final cell count of 2 to 4×10^6 ml.

Preparation of monolayers: Suspensions of PE cells were layered by pasteur pipette on to 7×20 mm "flying" glass coverslips in 1.2×7.4 cm glass tubes (WR tubes). Tubes were incubated at 37° C for 2 h and non-adherent cells removed by repeated washing with Dulbecco's PBS. Each coverslip then contained approximately 2×10^5 peritoneal exudate macrophages (PEMs).

Binding assay: Coverslips with adherent PE cells were layered with a suspension of the appropriate organism at the concentrations shown in the tables in Dulbecco's PBS containing Ca^{++} and Mg^{++} ions (Dulbecco's PBS + B) and incubated for 2 hours at 4° C. Non-attached organisms were washed away with Dulbecco's PBS and the coverslips air dried, heat fixed, and stained by the Gram method. Microscopic examination of the monolayers was performed and PEMs with two or more attached organisms were counted. A total of 200 cells were counted on each slide, and the results expressed as the percentage of cells binding organisms.

Inhibition studies: PEM cell monolayers were overlaid with solutions of various carbohydrates (hexoses, glucosamine, arabinose and 2 keto-3-deoxyoctonate (KDO), Sigma) and lipid A in Dulbecco's PBS + B. Lipid A was prepared from freeze dried *K. aerogenes* by phenol extraction (Poxton & Sutherland 1976). The concentration of inhibitors used was 10mM, as preliminary experiments had shown that this was the minimum inhibitory concentration for glucose and galactose. After fifteen minutes exposure to these materials at 4° C the monolayers were gently washed and the binding assay performed as described above. Bacterial concentrations for the inhibition study were those for which 15–30% binding had been observed in binding assays.

RESULTS

Binding of Various Gram Positive and Gram Negative Bacteria to PEMs

Binding assays were performed as described in Materials and Methods with a variety of Gram + ve and Gram -ve organisms. A working concentration of organisms was first determined that gave between 20 and 30% binding. At this level of binding the PE cell monolayers with attached

TABLE 1. Percent Binding of Organisms to Mouse PE Cell Monolayers and Inhibition by Glucose or Galactose

Organism	Numbers/ml	Percent binding	S. E.	Inhibitory sugars	
				Glucose	Galactose
<i>Str. viridans</i>	5×10^9	33.7	1.0	+	—
<i>Str. pyogenes</i>	5×10^{10}	19.5	0.8	+	—
<i>Str. pneumoniae</i>	up to 10^{11}	0			
<i>B. anthracoides</i>	3×10^9	23.0	0.8	—	—
<i>St. albus</i>	7×10^9	21.1	1.0	+	+
<i>St. aureus</i>	2×10^9	19.5	0.9	+	+
<i>E. coli</i>	7×10^8	21.0	1.0	+	+
<i>P. aeruginosa</i> (mucoid)	2×10^{10}	19.9	1.0	+	+
<i>P. aeruginosa</i> (non-mucoid)	1×10^{10}	20.9	1.0	+	+
* <i>C. parvum</i> (10390)	3×10^{10}	28.9	1.9	+	+

* Data from Ögmundsdóttir & Weir 1976.

TABLE 2. *Binding Inhibition - Klebsiella aerogenes Type 2*

<i>K. aerogenes</i> ($1-2 \times 10^{10}/\text{ml}$)	Percent binding (S. E.)	Inhibitory Sugars				
		Galactose	Glucose	Mannose	Glucosamine	Arabinose
Wild type 5055	18.1 (0.6)	-	+	-	-	-
Non-capsulated Mutant M2	19.0 (0.6)	+	+	-	+	-
Core Mutant M10B	19.1 (0.5)	-	+	-	+	-

organisms can readily be scored. The concentration of organisms required to achieve this binding level was usually between 10^9 and 10^{10} organisms/ml (see Table 1). In control experiments the formalin killing procedure was shown to have no effect on the binding ability of the organisms. *Str. pneumoniae* was the only organism tried that did not bind to the PEMs.

Inhibition of Binding by Monosaccharides and other Bacterial Wall Constituents

Using the working concentration of organisms, binding inhibition assays were performed with two commonly occurring cell wall sugars glucose and galactose. A sugar was regarded as inhibitory if it reduced binding to 60% of control values without inhibitor. Table 1 shows that glucose at a concentra-

TABLE 3. *Percent Binding of S. typhimurium Strains to Mouse PEMs and the Ability of Various Sugars and Cell Wall Constituents to Inhibit Binding. The Structure of the LPS of S. typhimurium, Wild Type and Mutants, is Shown in the Schematic Formula*

	O SOMATIC REPEAT UNIT	OUTER CORE		INNER CORE
	$\left[\begin{array}{ccc} \text{O Acetyl - Abe} & & \text{Glc} \\ & 1 \downarrow & 1 \downarrow \\ & 3 \downarrow & 4 \downarrow \\ & \text{Man - Rha - Gal} \end{array} \right]$	$\begin{array}{c} \text{GlcNac} \\ \text{Glc II - Gal I -} \end{array}$	$\begin{array}{c} \text{Gal II} \\ \text{Glc I - Hep II - Hep I} \end{array}$	$\begin{array}{c} \text{KDO} \\ \text{Lipid A} \\ \text{Ethanolamine} \end{array}$
Genotype	+	rfaK	Rc	rfaE
Chemotype	smooth	Rb		Re
STRAIN	SL 1542	SL 1096	SL 1099	SL 1102
INHIBITION BY:				
GLUCOSE	+	+	+	-
GALACTOSE	+	+	-	-
GLUCOSAMINE	+	-	-	-
RHAMNOSE	+	+	-	-
MANNNOSE	-	-	-	-
KDO	ND	ND	ND	+
LIPID A		+	+	+
ARABINOSE	-	-	-	-

+ inhibition, - no inhibition (see text).

Abbreviations Glc = glucose, Gal = galactose, Abe = abequeose, GlcNac = N-acetyl glucosamine, Man = mannose, Rha = rhamnose, Hep = L-glycero-D-mannoheptose, KDO = 2-keto-3-deoxyoctonate, ND = not done.
Concentration of organism $1-8 \times 10^9/\text{ml}$.

tion of 10mM was capable of inhibiting binding of all organisms except *B. anthracoides*; in contrast galactose was ineffective with this and two additional organisms *Str. viridans* and *Str. pyogenes*.

Similar binding inhibition assays were then performed with organisms of known cell wall constitution and with mutant strains with deficiencies of various sugars. Tables 2 and 3 show the results with 3 *K. aerogenes* and 4 *S. typhimurium* strains. The two *Klebsiella* mutants lacked capsule polysaccharides and M10B also had a defective lipopolysaccharide (LPS) core. Strain M10B contains only 0.2% (as % dry weight LPS) of galactose compared to between 20 and 30% in the wild type and mutant M2 (Poxton & Sutherland 1976). Table 2 shows that galactose has no inhibitory effect on the binding of the galactose deficient strain. Glucose which is present in a concentration of between 5 and 7% in all the strains is inhibitory with each of the organisms including the capsulated wild type. The bacterial cell wall also contains glucosamine which was inhibitory with the non-capsulated strain. Heptose and KDO are also present. The capsule contains both glucose and mannose but no galactose and mannose does not inhibit binding of any of the strains.

Table 3 shows the results of the inhibition assays with wild type *S. typhimurium* (SL 1542) and three mutants with deficiencies of sugars in the 0 repeat unit and outer and inner core, as indicated in the table. The ability of sugars to inhibit binding of a particular mutant is directly related to the presence of the sugar with the exception of rhamnose and mannose. The rough mutant SL 1102 appears to bind by means of its KDO and lipid A components. Lipid A is a glycolipid containing substituted galactose. The degree of inhibition is usually down to 40–60% of controls, but lipid A reduces the binding of SL 1102 to 12–15%.

DISCUSSION

The results indicate that microorganisms bind to adherent mouse PE cells by means of cell wall and capsular sugars. The binding sites on the plasma membrane of the PE cells appear to have specificity for a variety of cell wall sugars in that a mutant strain of *K. aerogenes* lacking in a cell wall sugar known to be involved in binding (galactose) still binds, presumably by means of its cell wall glucose component. The presence of a binding site for galactose is indicated by the ability of this sugar to partially inhibit the degree of binding of the *K. aerogenes* mutant known to contain galactose as a cell wall component (Table 2). The importance of these two sugars in the binding of a variety of Gram

+ve and Gram-ve organisms is indicated in Table 1.

The structure of the lipopolysaccharides of *Klebsiella (Enterobacter) aerogenes* is by no means as well defined as that of *Salmonella* species. The presence of large amounts of nonacetylated galactose in the wild-type and non-capsulated mutant (Poxton & Sutherland 1976) suggests that the 0 antigen is type 1 (Björndal *et al.* 1971). (The Lipid A of some strains resembles that of *Salmonella* and justified its use for inhibiting the SL 1102 *Salmonella* mutant as no *Salmonella* Lipid A was available).

With the *S. typhimurium* strains the schematic formula of which is shown in Table 3, the ability of sugars to inhibit binding is directly related to their presence in the 0 somatic repeat unit or the outer core. Mutants with missing sugars are not inhibited by those sugars. Mutant SL 1102 with only inner core components appears to bind by means of KDO and lipid A. The ability of lipid A to inhibit the binding of the other strains as well as SL 1102 can possibly be explained by its galactose content. The only anomaly is the inhibition by rhamnose of SL 1096 that should not express the sugar. This may be explained by the possibility that this mutant is "leaky" and synthesizing and expressing very small amounts of the wild type polysaccharide (Lehmann *et al.* 1973).

The inability of mannose to inhibit binding of *K. aerogenes* and the wild type *Salmonella* strain is noteworthy and a similar result was found in our earlier studies with *C. parvum* (Ögmundsdóttir & Weir 1976). It is known that in the 0 repeat unit of the wild type *Salmonella* (SL 1542) the mannose is linked at a branch point to abequose and in *Klebsiella* wild type capsules mannose is linked in the same way to glucuronic acid. The interaction with these sugars which are linked via hydrophilic and hydrophobic groups respectively may interfere with the expression of mannose as a potential binding sugar.

These results extend our earlier observations on *C. parvum* binding to mouse PE cells in which the importance of cell wall sugars in binding was clearly demonstrated (Ögmundsdóttir & Weir 1976). Work on the nature of the binding sites themselves indicates that they may be glycoprotein in nature (involving hydroxyl groups) in view of their susceptibility to proteolytic enzymes, β -galactosidase and periodate (Ögmundsdóttir *et al.* 1977). The requirement for Ca^{++} and Mg^{++} in binding has also been noted and appears also to be a requirement for the binding of unopsonized albumin coated oil particles to phagocytes and for the binding of lymphocytes to macrophages (Stossel 1975; Rosenthal *et al.* 1975).

In our view the binding of microorganisms to mouse PE cells, involving carbohydrates of the microbial cell wall and glycoprotein receptor sites on the macrophage plasma membrane, represents a primitive recognition mechanism enabling phagocytes to bind microorganisms prior to engulfment. It is likely that some organisms owe their pathogenicity to their ability to reduce the strength of binding by the presence of capsules that cover over sugars in the cell wall that would otherwise be involved in binding (and lead to phagocytosis). Other organisms may, by releasing capsular material, block the phagocyte binding sites and thus allow the organism to escape the attention of the phagocytic cell. The capsular polysaccharide of *Cryptococcus neoformans* can be shown to inhibit the binding of this yeast to the phagocyte. Encapsulated organisms attached at low rates and were engulfed more slowly than non-encapsulated yeasts (Kozel & Mastroianni 1976). In our studies the presence of a capsule did not appear to alter the degree of binding with the *Klebsiella* strains although the presence of the capsule did prevent galactose in the cell wall from attaching to the galactose binding site. We have not studied the stages that occur after initial binding and cannot therefore comment on the effects on virulence of the organism.

In other situations where virulence depends on the ability of microorganisms to attach to and penetrate gut epithelium, as is the case with *Salmonella* strains, then binding is a requirement for pathogenicity. *Str. mutans* appears to adhere to the tooth surface by means of carbohydrates (glucans) attached via a glycosyl transferase to a surface polysaccharide containing galactose and glucose (Gibbons & van Houte 1971). It seems possible that the more sugars involved the greater the chance of attachment to, penetration and invasion of, host cells (particularly those cells with fewer binding sites). Table 3 confirms this suggestion in that the smooth virulent strain of *S. typhimurium* (SL 1542) binds to the phagocytic cells (and therefore presumably to other cells) by more sugars than any of the less virulent mutants. Although *S. typhimurium* wild type (LT2) appears to be initially more slowly phagocytosed than rough strains (Nakano & Saito 1968), phagocytosis and digestion is not a necessary sequel to binding (see Smith 1977). We suggest that the binding phenomenon described may have been the forerunner of the more effective binding mechanisms that involve cell bound opsonins and complement factors and would perhaps have provided a degree of protection from at least some potentially pathogenic microorganisms. Whether or not binding interaction by macrophages such as we have described extends to other forms of macro-

phage adherence remains to be elucidated. It is conceivable that interaction of this type may be responsible for macrophage recognition and attachment to a variety of carbohydrate containing surfaces such as transformed or altered tissue cells and carbohydrate antigenic determinants (Weir & Ögmundsdóttir 1977).

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Stimulation of phosphatidylinositol turnover in the macrophage plasma membrane: a possible mechanism for signal transmission

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Summary. The effect of various stimuli on the rate of phosphatidylinositol turnover by mouse peritoneal macrophages was studied by measuring the incorporation of *myo*-[2-³H]-inositol. It was found that the macrophage-activating agents endotoxin and *Corynebacterium parvum* caused an increase in phosphatidylinositol turnover whilst inert particles (*Staphylococcus albus*, latex and carbon) had no effect even though they were phagocytosed. The stimulation by *C. parvum* was dependent on the presence of T cells. T lymphocytes from normal mice and mice immunized with *C. parvum* were equally effective and it was found that both normal and immune T cells could be stimulated by *C. parvum* as indicated by an increased uptake of [6-³H]-thymidine. Supernatants from normal or immune spleen cells cultured with and without *C. parvum* were ineffective and could not replace intact T cells, indicating that cell to cell contact is required or a labile factor that acts over a short range. The time course of stimulation of phosphatidylinositol turnover by macrophages was studied using endotoxin. The rate of turnover increased slowly over a few hours and was still rising at 6 h but decreasing again at 24 h. The increase in bacteriostatic activity against *Listeria monocytogenes* by macrophages exposed to endotoxin

took longer to develop and was more marked at 24 h than at 4 h. The differences between pathways leading to phagocytosis or chemotaxis and those resulting in activation are discussed.

INTRODUCTION

The biochemical pathways that are set in motion by substances that act on the macrophage plasma membrane are poorly understood although the widely recognized effects of interaction such as 'macrophage activation' and phagocytosis are of importance in antibacterial and tumour immunity (Keller, 1977). The transmission of signals across the plasma membrane frequently involves ion fluxes and can be mediated by second messengers such as cyclic nucleotides. Interaction with a cell surface receptor often leads to an early increase in the turnover of the phosphorylinositol headgroup of membrane lipid phosphatidylinositol and has been found to occur in many cell types including lymphocytes (Maino, Hayman & Crumpton, 1975; Michell, 1975). A model has been proposed by Michell, Jones & Jafferji (1977) where the surface interaction leads to a transduction step of increased phosphatidylinositol turnover which causes release of membrane bound Ca²⁺ and opening of membrane Ca²⁺ gates. This is followed by an amplification step of increasing intracellular concentration of Ca²⁺ which triggers intracellular responses. The rate of phosphatidylinositol turnover is reflected in the rate

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of uptake of ^{32}P , and labelled inositol (Michell, 1975) and in this work we have measured *myo*-[2- ^3H]-inositol incorporation in mouse macrophages exposed to the macrophage activating agents bacterial endotoxin (Alexander & Evans, 1971) and *C. parvum* (Ghaffar, Cullen, Dunbar & Woodruff, 1974) and compared their effects to those of particles that are phagocytosed but do not cause activation (carbon, latex, and *S. albus*).

MATERIALS AND METHODS

Animals

C3Hf/Bu mice, SPF, age 6–10 weeks were obtained from the departmental breeding colony.

Reagents

Myo-[2- ^3H]-inositol and [6- ^3H]-thymidine were obtained from the Radiochemical Centre, Amersham. Lipopolysaccharide W (endotoxin) from *E. coli* 055:B5 and latex particles (0.81 μm) came from Difco Laboratories, Detroit. A stock suspension of colloidal carbon was a kind gift from the Department of Pathology, University of Edinburgh. *Corynebacterium parvum* (strain 10390), originally obtained from the National Collection of Type Cultures, Colindale, was grown anaerobically in horse digest broth containing 3% glucose. *Staphylococcus albus* was grown aerobically in nutrient broth. The bacteria were harvested in log phase, killed by 24 h exposure at 4° to 0.5% formalin in saline and washed four times with saline. *Listeria monocytogenes* was grown aerobically in nutrient broth and washed three times in Dulbecco's phosphate-buffered saline (PBS) before use. Bacteria were counted in a Neubauer chamber 0.01 mm depth. Foetal calf serum was obtained from Sera-lab, Crawley Down, Sussex. The serum was heat-inactivated for 30 min at 56° before use.

Harvesting of peritoneal exudate cells and culture of macrophages

The mice were injected i.p. with 2.5 ml Dulbecco's PBS containing 10 i.u./ml of heparin. After gentle massage the fluid was withdrawn by means of a syringe. Peritoneal fluid from eighteen to twenty-four mice was pooled for each of the inositol-uptake experiments. The cells were kept on ice, centrifuged at 4°, 250 g for 7 min and resuspended in Eagle's medium (MEM, Wellcome Research Laboratories, Beckenham) to a con-

centration of approximately $2.5\text{--}3.0 \times 10^6$ cells/ml. This suspension was transferred into 13 \times 150 mm culture tubes in 3 ml amounts and incubated on a slope for 90 min at 37°. The tubes were then washed seven times with Dulbecco's PBS containing Ca^{2+} and Mg^{2+} ions (Dulbecco's PBS+B) to remove non-adherent cells and the adherent cells (macrophages) were re-incubated overnight at 37° in Eagle's medium containing 5% foetal calf serum, before use in the assay.

An aliquot of the cell suspension was put on to two 'flying' coverslips which were incubated and washed in the same way as the tubes and the adherent cells counted by inverting them on to a haemocytometer (Improved Neubauer). Each tube usually contained approximately 3×10^6 adherent cells; of these approximately 95% were macrophages as shown by phagocytosis of antibody-coated *C. parvum* on coverslip preparations. The degree of phagocytosis of test particles used in the inositol-uptake experiments was tested in parallel cultures on coverslips which were gram-stained and examined microscopically or left unfixed and unstained and counted using phase contrast optics in the case of latex particles.

Immunization with *C. parvum*

C. parvum was injected i.p. into the mice at a dose of 3×10^{10} organisms (0.7 mg dry weight) in 0.1 ml saline. Spleens from these animals were used 14 days later at which time there was a marked splenomegaly and a high titre of serum antibodies. The spleen cells from these animals will be referred to as immune cells.

Preparation of spleen cell cultures and separation of T cells

Spleen cell suspensions from normal and immune mice were prepared by pushing one to two spleens gently through a sterile wire mesh. The cells were centrifuged at 4° for 7 min at 250 g and red blood cells were removed by resuspending the cells in a buffer composed of 0.155 M NH_4Cl , 0.1 mM EDTA and 0.01 M KHCO_3 for 4 min. The cells were centrifuged and finally resuspended in tissue culture medium. For the production of culture supernatants the spleen cell suspension was adjusted to 2×10^6 cells/ml and cultured for 4 h at 37° in 10 ml of Eagle's medium without serum in tissue culture bottles in the presence or absence of *C. parvum* at a final concentration of 2×10^8 bact./ml. The supernatants were harvested by centrifuging at 500 g for 15 min at 4° and centrifuging the resulting supernatant again at 2000 g for 20 min at 4°.

The supernatants were stored overnight at 0° before use.

T cells were separated from a spleen cell suspension on a nylon wool column as described by Hunt (1978).

Preparation of lipid carrier from livers

The lipids were extracted from 25 g of homogenized mouse livers with 50 ml of a 2:1 mixture of chloroform and methanol for 20 min at 37°. After the addition of 25 ml of 0.5 M MgCl₂ the mixture was centrifuged for 10 min at 300 g. The upper aqueous phase was discarded and the organic phase withdrawn from underneath the compressed liver tissue. The organic phase was then equilibrated against 0.5 M MgCl₂ in the same way as before, the aqueous phase discarded again and residual water removed from the organic phase with anhydrous Na₂SO₄. The organic phase was finally evaporated to dryness under vacuum and redissolved in chloroform-methanol to give a concentration of extract from 2 g of liver per ml and stored at -20°. For use in the assay the extract was diluted 1:10 in chloroform-methanol.

Assay for uptake of myo-[2-³H]-inositol

After the macrophages had been incubated overnight with Eagle's medium with 5% foetal calf serum they were washed with Dulbecco's PBS + B and all subsequent steps performed in Eagle's medium without serum. The cells were then incubated with various test particles and substances usually for 4 h at 37°, 2 µCi of myo-[2-³H]-inositol were added in 0.1 ml of medium to each tube one hour before the end of the culture period. The cell metabolism was then stopped by putting the tubes into ice. They were then washed 7 times with Dulbecco's PBS and 5 ml of chloroform-methanol added to each tube. The lipids were extracted for 20 min at 37° with vigorous shaking on a Rotamixer every 5 min. Two millilitres of liver lipid extract were then added as a carrier and 3 ml of 0.5 M MgCl₂ and mixed well. The phases were separated by centrifugation and the organic phase equilibrated once more against 0.5 M MgCl₂ as described above. The organic phase was then transferred into liquid scintillation counting vials and evaporated to dryness at 40° under a stream of N₂. Toluene-based scintillation fluid containing PPO and POPOP was added and radioactivity counted in a Packard Scintillation spectrometer.

Thin layer chromatography separation of the lipid phase confirmed that all the radioactivity was associated with phosphatidylinositol.

Assay of DNA-synthesis by T lymphocytes

T lymphocytes, separated on a nylon wool column were incubated in flat bottom microtitre tissue culture plates (Sterilin, Teddington, Middlesex) in 0.2 ml of Eagle's medium with 10% foetal calf serum and 0.04 mM 2-mercaptoethanol. The microtitre plates had been prepared by incubating them for 90 min with peritoneal exudate cells. The non-adherent cells were washed away leaving the macrophages in a final number of approximately 5% of the number of T lymphocytes. *C. parvum* was added in 10 µl amounts to give the final ratios of 100 and 500 bacteria per cell. These microtitre plates were incubated for 3 days at 37°, 4 h before the end of the culture period the medium was gently removed and replaced by medium containing [6-³H]-thymidine, 0.2 µCi per well. The non-adherent cells were then precipitated on to Whatman glass fibre filters with 10% TCA in a sampling manifold (Millipore Corporation, Bedford, Massachusetts). Radioactivity was then counted in a Packard Scintillation spectrometer as described above.

Bacteriostatic effect of macrophages against Listeria monocytogenes

Peritoneal exudate macrophages were cultured on 'flying' glass coverslips in the manner described above. The coverslips were incubated with *E. coli* lipopolysaccharide (60 µg/10⁶ cells) in medium without serum for 4 h or 24 h. The cells were then washed and overlaid with a suspension of *Listeria monocytogenes* in Eagle's medium containing 15% foetal calf serum but no antibiotics to give a ratio of 7-10 bact./cell. The coverslips were then incubated for 30 min at 37° after which they were washed six times with Dulbecco's PBS + B. Half of the coverslips were then air-dried, heat-fixed and gram-stained and the remaining half reincubated with Eagle's medium with 15% foetal calf serum and no antibiotics for 4 h and then processed in the same way. The coverslips were examined microscopically, the number of cells per field, the number with ingested bacteria and the number of intracellular bacteria were counted; twelve to eighteen fields were counted on each slide. For comparison of bacteriostatic effect the number of intracellular bacteria per total number of cells was calculated as this takes account of the proportion of cells containing bacteria. This figure was used to calculate the growth ratio of *L. monocytogenes* by dividing the number at 4 h by the number at 30 min. The relative bacteriostatic effect of lipopolysaccharide-treated cells *v.* controls was calculated as growth ratio in controls/growth ratio in treated cells.

RESULTS

Turnover of phosphatidylinositol by macrophages exposed to various stimuli

Macrophage monolayers were exposed to various stimuli in serum-free medium *in vitro* and the effect on the uptake of *myo*-[2-³H]-inositol measured. The results are shown in Table 1, which also gives the concentrations of substances and particles used.

It can be seen that both endotoxin and *C. parvum* cause an increase in the uptake of labelled inositol indicating an increased rate of phosphatidylinositol turnover. Endotoxin has a stimulatory effect both with and without T lymphocytes whilst *C. parvum* is effective only in the presence of T cells. The ratio of T cells to macrophages was 1:5.

All the test particles were ingested by macrophages, *C. parvum* was taken up by 33% of the cells, latex particles by 45%, carbon particles by 16% and *S. albus* by 23%, all during 4 h in serum-free medium. No difference was observed in the number of adherent cells at the end of the incubation period between control and treated cultures.

Requirement for T lymphocytes for stimulation of macrophages by *C. parvum*

The nature of the requirement for T cells for the

stimulation of phosphatidylinositol turnover by macrophages caused by *C. parvum* was investigated further. The requirement seemed absolute and even prolonged exposure (up to 4 days) to *C. parvum* without T cells had no stimulatory effect.

Table 2 shows that T cells from animals immunized 14 days previously with *C. parvum* were no more effective than T cells from normal animals.

In order to test whether the effect of the T cells was mediated by a soluble factor, supernatants were prepared from spleen cells cultured for 4 h under conditions equivalent to those used in the assay. All the supernatants were inactive regardless of whether they were derived from cultures of normal or immune spleen cells and whether or not they had been exposed to *C. parvum* during the culture period (see Table 3).

It can also be seen that the supernatants failed to substitute for intact T cells when the macrophages were treated with *C. parvum*.

The presence of T cells had no effect on the degree of uptake of *C. parvum* by macrophages. Small cells were seen to cluster around macrophages at the end of the culture period but after the final washing procedure none of these remained and the number of adherent cells was the same as in control cultures. It is therefore unlikely that T cells contributed to the radioactivity measured.

Table 1. The effect of various stimuli on the uptake of *myo*-[2-³H]-inositol by macrophages. The figures represent means of pooled data from two to three experiments, each done in triplicate. Significance of differences was determined using Student's *t* test (two-tailed). *P* values < 0.05 are considered significant, n.s. = not significant

Macrophages exposed for 4 h to:	Uptake of <i>myo</i> -[2- ³ H]-inositol, percentage of unstimulated controls		In presence of T cells	
Endotoxin (<i>E. coli</i> , 055:B5 Difco), 60 µg/10 ⁶ cells	129.1	SE 4.7 <i>P</i> < 0.001	129.7	SE 9.5 0.01 < <i>P</i> < 0.02
<i>Corynebacterium parvum</i> (10390) 100 bact./cell	106.3	SE 5.4 n.s.	122.9	SE 6.5 0.02 < <i>P</i> < 0.025
Latex particles (Difco) 500 particles/cell	93.9	SE 4.9 n.s.	106.7	SE 10.3 n.s.
Carbon particles (OD 0.36)	94.1	SE 2.1 n.s.	96.7	SE 5.3 n.s.
<i>S. albus</i> 500 bact./cell	96.6	SE 4.0 n.s.	95.5	SE 9.5 n.s.
Eagle's medium without serum	100.0	SE 4.4-6.0	102.1	SE 2.5-6.5 n.s.

Table 2. The effect of the presence of immune T cells on the stimulation of phosphatidylinositol turnover by macrophages. The figures represent means of pooled data from four experiments, each done in triplicate. *P* values determined using two-tailed Student's *t* test, *P* < 0.05 significant, n.s. = not significant

Macrophages exposed for 4 h to:	Uptake of <i>myo</i> -[2- ³ H]-inositol, percentage of unstimulated controls		
<i>Corynebacterium parvum</i> (10390) 100 bact./cell	110.9	SE 6.3	n.s.
<i>Corynebacterium parvum</i> and immune T cells	118.9	SE 6.2	0.02 < <i>P</i> < 0.025
Immune T cells	102.2	SE 4.2	n.s.
Eagle's medium without serum	100.0	SE 4.3	

As normal and immune T cells were equally effective it was of interest to know whether *C. parvum* could stimulate T cells from normal as well as immune animals. This was done by measuring the uptake of [6-³H]-thymidine by T cells cultured in the presence of a small proportion of macrophages and exposed to two different doses of *C. parvum*.

As shown in Table 4 both normal and immune T cells responded to *C. parvum* by an increased uptake of labelled thymidine, the immune cells had a higher level of uptake than the normal cells in the absence of *C. parvum*. The higher ratio of bacteria to cells is equivalent

to that used in the assay for phosphatidylinositol turnover. The response of the T cells was absolutely dependent on the presence of a low percentage of macrophages (5%). *C. parvum* was not seen to bind directly to T cells in gram-stained smears.

Time course of stimulation of phosphatidylinositol turnover

The time course of stimulation of phosphatidylinositol turnover by macrophages caused by endotoxin was studied by measuring the uptake of *myo*-[2-³H]-

Table 3. The effect of supernatants from normal and immune spleen cells cultured with and without *C. parvum* on the rate of phosphatidylinositol turnover by macrophages in the presence and absence of *C. parvum*. The figures are the means of pooled data from two to four experiments, each done in triplicate. *P* values determined using two-tailed Student's *t* test, *P* < 0.05 significant, n.s. = not significant

Macrophages exposed for 4 h to:	Uptake of <i>myo</i> -[2- ³ H]-inositol, percentage of unstimulated controls			
			In presence of <i>Corynebacterium parvum</i>	
Supernatant from normal spleen cells	90.8	SE 4.7 n.s.	98.9	SE 3.7 n.s.
Supernatant from normal spleen cells cultured for 4 h with <i>C. parvum</i>	109.2	SE 8.7 n.s.	112.0	SE 4.8 n.s.
Supernatant from immune spleen cells	102.4	SE 8.5 n.s.	92.1	SE 6.4 n.s.
Supernatant from immune spleen cells cultured for 4 h with <i>C. parvum</i>	104.9	SE 9.0 n.s.	91.0	SE 8.9 n.s.
Eagle's medium without serum	100.0	SE 3.4-3.9	110.9	SE 5.8-11.6 n.s.

Table 4. The effect of *C. parvum* on the uptake of [6-³H]-thymidine by T cells from normal mice and mice immunized 14 days previously with *C. parvum*. *P* values determined using two-tailed Student's *t* test, *P* < 0.05 significant, n.s. = not significant.

<i>Corynebacterium parvum</i>	Uptake of [6- ³ H]-thymidine, counts per minute			
	Normal T cells		Immune T cells	
None	450.8	SE 129.4	1008.0	SE 72.4
100 bact./cell	1372.7	SE 141.3 <i>P</i> < 0.001	1251.4	SE 53.4 0.01 < <i>P</i> < 0.02
500 bact./cell	1743.0	SE 328.0 0.005 < <i>P</i> < 0.01	2826.7	SE 310.5 <i>P</i> < 0.001

inositol after 1, 4, 6 and 24 h of incubation. The labelled inositol was always added 1 h before the end of the culture period.

As can be seen in Fig. 1 there is a slight, but not significant, increase in uptake after 1 h (0.05 < *P* < 0.10). The turnover is significantly increased after 4 h and still rising at 6 h. At 24 h the uptake has gone back to about the same value as at 4 h.

The time course of development of increased anti-bacterial activity of macrophages exposed to endotoxin is also indicated on Fig. 1, for comparison. This is clearly a much slower process and the bacteriostatic capacity is not markedly raised until after 24 h when the response in phosphatidylinositol turnover has started to fade away.

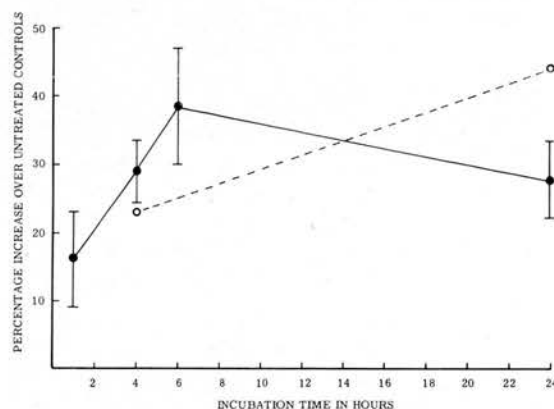


Figure 1. The filled circles for myo-[2-³H]-inositol uptake represent means of pooled data from three to four experiments, each done in triplicate, vertical bars = SE. The open circles for bacteriostatic activity depict data from one representative experiment performed in triplicate.

In the experiment depicted in Fig. 1, the growth ratios of *L. monocytogenes* were as follows: After 4 h exposure to endotoxin it was 1.41 but 1.73 in parallel control cultures, after 24 h treatment with endotoxin it was 2.05 as compared with 2.96 in the controls.

DISCUSSION

The results presented in this paper indicate that certain stimuli but not others cause an increase in the turnover of phosphatidylinositol in the cell membrane of the macrophage. This response is not linked either to the binding event or to the process of phagocytosis since some of the particles tested (*viz.* latex, carbon and *S. albus*) had no effect on the uptake of myo-[2-³H]-inositol although they were phagocytosed. These results contrast with those of Graham, Karnovsky, Shafer, Glass & Karnovsky (1967) working with granulocytes and caseinate-induced macrophages from guinea-pigs who found increased incorporation of inorganic phosphorous into phosphatidylinositol following phagocytosis of particles or exposure to certain surface active agents. Induced macrophages after phagocytosis of inert particles have been shown to behave differently from normal macrophages in other ways including production of enzymes such as acid hydrolases and neutral proteases as well as showing enhanced metabolic activity (Davies, Bonney, Humes & Kuehl, 1977).

It is noteworthy that endotoxin can stimulate turnover directly whilst *C. parvum* requires the presence of splenic T lymphocytes. Normal T cells were as effective as T cells from animals immunized with *C. parvum* and the organisms could initiate DNA synthesis even

in the cells from unimmunized animals, suggesting either prior sensitization or a non-specific mitogenic effect. Culture supernatants of lymphocyte suspensions exposed to *C. parvum* were ineffective in inducing increased phosphatidylinositol turnover irrespective of whether they came from immunized or normal mice. These supernatants were also inactive when added to the macrophages with *C. parvum*. This indicates that either cell to cell contact is required between the lymphocyte and macrophage or that a factor is produced that acts only at a short range or is a labile molecule. *C. parvum* does not bind directly to T lymphocytes nor are lymphocytes attached to macrophages at the stage that *myo*-[2-³H]-inositol uptake is assayed. They can however be seen to cluster round macrophages in the first stage of the test and are later washed away. The degree of uptake of *C. parvum* by macrophages is not influenced by the presence of T cells. The events that lead to increased phosphatidylinositol turnover in the macrophage membrane seem likely to depend on surface attachment of *C. parvum* to the macrophage followed by its presentation to T lymphocytes. This then leads to lymphocyte stimulation with consequent activation of macrophages.

Attempts to activate purified normal macrophages *in vitro* with *C. parvum* have been unsuccessful and Christie & Bomford (1975) found that only oil-induced macrophages could be rendered cytotoxic by exposure to *C. parvum in vitro* whereas for normal macrophages the presence of a large number of sensitized T cells was required. On the other hand activation can be achieved *in vivo* in thymectomized animals (Ghaffar, Cullen & Woodruff, 1975).

It seems likely that *C. parvum*, *S. albus* and *E. coli*-lipopolysaccharide all bind to the macrophage plasma membrane by the same mechanism based on recognition of carbohydrates (Freimer, Ögmundsdóttir, Blackwell, Sutherland, Grahame & Weir, 1978; Weir & Ögmundsdóttir, 1977) but only the lipopolysaccharide can stimulate the macrophages directly. Wahl, Wahl, Mergenhagen & Martin (1974) found that the lipid moiety was required for the stimulation by endotoxin of collagenase production by macrophages. It is possible that the stimulation by endotoxin occurs by a mechanism similar to that suggested for the action of cholera toxin where the part of the molecule that binds to the plasma membrane initially is itself inactive but is thought to release the active component into the membrane (Cuatrecasas, Bennett, Craig, O'Keefe & Sahyoun, 1976).

Many stimuli resulting in enhanced metabolic activ-

ity of cells with involvement of increased turnover of phosphatidylinositol also cause an increase in cGMP levels whilst the concentration of cAMP stays unchanged or decreases (Michell, 1975). Muscarinic cholinergic stimulation is a well studied example and the increase in cGMP following cholinergic stimulation of pancreatic acinar cells has also been found to be dependent on the presence of Ca²⁺ (Christophe, Frandsen, Conlon, Krishna & Gardner, 1975). Rabbit alveolar macrophages can be activated by the ionophore A-23187, which facilitates transfer of Ca²⁺ and Mg²⁺ across membranes (Hand, King, Johnson & Lowe, 1977) and cGMP induces the release of lymphocyte activating factor (Diamantstein & Ulmer, 1976). In addition, it has been concluded that the action of MIF (migration inhibition factor) on macrophages does not lead to an increase in cAMP (Higgins, Winston & David, 1976; Pick, 1977).

In contrast, stimulation of the contractile elements of phagocytes (as occurs with the inert particles in the present study), reflected in the rapidly appearing phenomena of chemotaxis and phagocytosis, does not involve phosphatidylinositol turnover and may depend upon a mechanism involving membrane permeability changes more akin to the action of acetylcholine on the motor end plate of striated muscle. Naccache, Showell, Becker & Sha'afi (1977) have recently shown that a chemotactic agent leads to a large and rapid (within minutes) increase in the permeability of the plasma membrane of rabbit polymorphonuclear leucocytes to Na⁺ followed by a smaller enhancement of K⁺-influx and Na⁺-efflux. There is also an increase in membrane permeability to Ca²⁺ and the intracellular exchangeable Ca²⁺ pool.

An increased phosphatidylinositol turnover in comparison is a more slowly developing process and although it can first be detected in several tissues in about 10 min after applying the stimulus, it increases substantially over a much longer period (Maino *et al.*, 1975; Michell, 1975). In our system endotoxin causes a slight but not significant increase in uptake of *myo*-[2-³H]-inositol after one hour, the rate of uptake is still increasing after 6 h but has decreased again after 24 h. The bacteriostatic effect of macrophages following exposure to endotoxin takes even longer to appear and was more marked after 24 h than after 4 h. In general, bacteriocidal, cytotoxic and other changes associated with macrophage activation *in vitro* do not appear until after 24–48 h (Diamantstein & Ulmer, 1976; Churchill, Piessens, Sulis & David, 1975).

Thus the slowly developing effects of endotoxin and

C. parvum leading to macrophage activation may be explained by the phosphatidylinositol turnover model whilst the rapidly occurring process of phagocytosis appears to involve a different pathway. The detailed mechanisms of these processes await further investigation.

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